Introduction

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One of the primary objectives of agriculture is to provide the food and fibre needs of human beings. These needs increase as the population increases. The 20th century has witnessed a dramatic change in rate of growth of world demand for food due to population explosion. The world population in 1990 was 5.3 thousand billion. It is expected to be 6.3 thousand billion in 2000, and by 2025, it is expected to reach 8.5 thousand billion. These growth rates will require an increase in food production of about 40 to 50 percent over the next thirty to forty years. Growth in crop production can come from increases in arable land, cropping intensity and yield per unit area of cropped land. But as the area of cultivable land is more or less fixed, it becomes highly essential to push agriculture farther into marginal lands lying under uncultivable conditions due to soil salinity, alkalinity or other environmental stresses.

Salinity is one of the major environmental problems constraining food production by affecting large areas of otherwise arable land surface (Epstein, 1980).

Saline affected area around the globe is estimated to be 1 billion hectares. In India, about 8.1% of the total geographical area is affected by salinity (Rao, 1990). Gujarat alone has around 7 lac hectares of uncultivable land due to salinity.

Salinity refers to the occurence of various soluble salts in soil or water in concentrations that may interfere with the growth of plants. Though sodium

chloride (NaCl) is sometimes the most predominant salt present, the term salinity includes chlorides, sulphates and bicarbonates of sodium, magnesium and potassium (Chapman, 1975; Abrol, 1986). There are multitude of ways by which concentration of these salts can be expressed, but the preferred expression by physiologists and soil scientists is electrical conductivity (EC) stated as decisiemens per meter (dS/m) or millimhos per centimeter (mmhos/cm). According to U.S. Salinity laboratory recommendations a soil with an electrical conductivity of 4dS/m or if all the dissolved salt is sodium chloride with an ionic concentration of 44 millimho or more can be considered as saline.

To combat the increasing problem of salinity there have been two major approaches, the technological approach and the biological approach. The technological approach makes use of advances made in water and soil management, irrigation methodology (Pasternak, 1982) and desalination of salts (Gale, 1982). But this technology of combating salinity is extremely costly, requiring large expenditures of energy to reclaim land and maintain salt balances. It also involves such energy intensive procedures as recontouring land by deep-ripping and land planning, the installation of agricultural drains, the pumping and conveyance of irrigation water, and even the desalination of water (Downton, 1984). As the cost of energy continues to rise, it is increasingly clear that other alternatives must be found. Furthermore to combat salinity there is a need to reuse water with increased salt loads arising from agricultural drains, as water is a valuable resource in its own right which can be used to grow more salt tolerant crops or even biomass for energy conversion. Ultimately, the ability of

the crop itself to tolerate a given level of salinity becomes paramount in the management of water and soil resources. For this reason there has been an upsurge of interest towards tailoring crop plants to suit more saline environments. This is the biological approach which includes introgression of useful agronomical traits from the salt tolerant wild plant species. The existence of biological variability for salt tolerance within the species of concern can be exploited and tolerant agronomic crops by selective breeding can be developed. But these traditional methods of plant breeding are extremely labour intensive and time consuming (Shannon, 1982). Also, whole plant breeding system have met with limited success in improving the response of crops to saline stress (Epstein, 1980; Norlyn, 1980). It is now well established that plant tissue and cell culture techniques can be successfully employed to develop salt tolerance in crops as well as to study the cellular basis of salinity tolerance in plants (Rains *et al.*, 1986). Zenk (1974) reported for the first time the isolation of salt-resistant cell line from haploid cells of *Nicotiana sylvestris*. The resistant strain was able to grow in 1% NaCl at about 50% of the control rate, while no growth occurred at 1% NaCl for the non-selected cells. Since then, there have been numerous reports on the *in vitro* production of salt tolerant plant species (Vajrabhaya *et al.*, 1989; Freytag *et al.*, 1990; Winicov, 1991; Ibrahim *et al.*, 1992; Sumaryati *et al.*, 1992; Gulati and Jaiwal, 1993b).

The suitability of cell culture technique for plant improvement is because of the relative ease with which a mutant cell line can be obtained and regenerated to give a plant with same characters as the cell line. Also, large population is

available for selection, selection pressure can be applied more effectively and increase in genetic variability (somaclonal variation) is often associated with maintenance *in vitro*. A unique opportunity also exists to study cellular level mechanisms and functions of salt tolerance. Complications due to differences in morphology and stages of development are reduced when using cell cultures because of the relatively homogenous and undifferentiated nature of the cultured cells. With cell culture methods the environment and nutrient conditions can be uniformly and precisely controlled. A large number of cells can be screened rapidly in a relatively small area. Genetically similar material can be used (i.e. selected and unselected cells which differ in the degree of tolerance) so that observations will be related to the tolerance of the cells. Traits can be selected at the cell level and salt tolerance or somaclonal variability for that trait can be evaluated in regenerated plants or their progeny (Rains *et al.*,1980). The unique advantages, the methodologies and accomplishments of the *in vitro* production of salt-tolerant plants have been reviewed by many authors (Meredith, 1984; Chandler and Thorpe, 1986; Rains *et al.*, 1986; Collin and Dix, 1990; Nabors, 1990; Tal, 1990, 1993; Dix, 1993).

Salinity causes a number of adverse metabolic changes, leading to retarded growth *in vitro*. Decrease in growth (in terms of fresh weight and dry weight) of non - selected cells with increasing concentration of salinity has been reported by Pua and Thorpe (1986), and Muralitharan *et al.* (1990). Gulati and Jaiwal (1992), have also observed a reduction in dry weight with increasing concentration of salinity. A general trend of decreased callus growth with

increasing levels of NaCl in the medium has been reported by Bhaskaran *et al.* (1983); Janardhan Reddy and Vaidyanath (1986) and Li (1990). Growth inhibition by saline stress is commonly accepted to be due to lowering of the water potential of growth media caused non - specifically by dissolved excess ions (Flowers *et al.*, 1977; Greenway and Munns, 1980). Prakash and Sarin (1993) observed a decrease in water and solute potential of cell lines of *Cajanus cajan* subjected to increasing NaCl concentrations.

NaCl has also been found to affect the genetics of the cells. Cytogenetic studies by Kononowicz *et al.* (1990), have shown that high levels of NaCl induce polyploidization. Alterations involving chromosome number and structure represents one possible mechanism of permanent genetic change which could be involved in the adaptation of stable tolerance by cultured plant cells.

The activity of a number of key enzymes has shown to be increased or decreased by NaCl salinity. Subhashini and Reddy (1990) have reported an increase in the activities of peroxidase, polyphenoloxidase, alkaline inorganic pyrophosphatases and glutamate dehydrogenase under salinity in callus cultures of salt tolerant and salt susceptible rice cultivars. Studies of Gossett *et al.* (1994) with callus cultures of salt tolerant cultivar of cotton grown on saline medium showed significant increases in superoxide dismutase, catalase, ascorbate peroxidase, peroxidase and glutathione reductase activities as compared to callus tissue grown on NaCl free medium. The NaCl - induced increase in the activity of these enzymes indicate that the callus has higher capacity for scavenging and dismutating superoxide, an increased ability to decompose H_2O_2, and a more

active ascorbate - glutathione cycle when grown on media amended with NaCl. Thus the cells are protected from the potential cytotoxic effects of activated oxygen species which include damage to lipids (Wise and Naylor, 1987), nucleic acids (Imlay and Linn, 1988), and protein (Davies, 1987). A decreased proline oxidase activity has been observed in salinized callus of wild species of *Lycopersicon pennellii* (Rus Alvarez and Guerrier, 1994).

Intracellular levels of ions such as Na^+, K^+, Cl^-, Mg^{2+} and Ca^{2+} are also implicated in playing an important role in determining the plant response to excess of salt levels. Salt stress induces severe problems for plant growth (Staal *et al.*, 1991). Ion imbalance (especially the K^+/Na^+ ratio), water stress and ion toxicity (elevated concentrations of cytoplasmic Na^+ and Cl^-) may lead to reduced growth and eventually to plant death. Plant species differ greatly in their strategy and capability to cope with these problems (Flowers *et al.*, 1977; Greenway and Munns, 1980). It is generally envisaged that a primary consequence of exposing plants to high NaCl environment is the rapid accumulation of Na^+ and Cl^- in the cytoplasm. Sodium accumulation accompanied by loss of K^+ in response to increased levels of NaCl in the medium has been reported by Paek *et al.* (1988), Gulati and Jaiwal (1992), and Blits *et al.* (1993).

Since the cytoplasm is very susceptible to the inhibitory effects of Na^+ and Cl^-, evacuation of these ions from the intracellular compartment is a priority for survival and growth. Efflux of these ions across the plasma membrane is mediated by secondary active transporters presumably an Na^+/H^+ antiporter and a Cl^- channel (Braun *et al.*, 1989; Tester, 1990) that utilize the H^+

electrochemical gradient produced by the activity of the plasma-membrane localized H^+-ATPase. Data of Niu *et al.*(1993) provide evidence that activation of plasma-membrane H^+-ATPase is in response to a saline environment involving m-RNA accumulation. There are also several reports to show that in salt- tolerant and halophytic species a Na^+/H^+ antiport system can be induced to drive Na^+ accumulation in the vacuole (Blumwald and Poole, 1985; Garbarino and DuPont, 1988; Matoh *et al.*, 1989; Staal *et al.*, 1991). This mechanism helps to sequester Na^+ from the cytoplasm into the vacuole.

It has been known for a few decades that Ca^{2+} attenuates the inhibition of cell growth caused by NaCl (Lauchi and Schuber, 1989). This is attributable in part to a role of calcium in the preservation of plasma membrane integrity (Cramer *et al.*, 1987). Additionally, salt stress results in higher cytosolic Ca^{2+} activity (Lynch and Lauchli, 1988). Fine control of Ca^{2+} concentrations in different intracellular compartments is required because cytosolic Ca^{2+} greatly influences the metabolic processes of a cell owing to its role as a second messenger in signal transduction (Poovaiah and Reddy, 1987). Ca^{2+}-ATPases by being involved in Ca^{2+} transport are therefore of considerable importance in salt adaptation. Results of Perez - Prat *et al.*(1990), have indicated that NaCl adapted cells have higher levels of the Ca^{2+}-ATPase mRNA.

In the recent years, there has been an increased interest in studying the role of polyamines in various stress - induced responses of plants (Smith, 1985). Polyamines appear to be ubiquitous in living cells and have been implicated in a variety of regulatory processes ranging from promotion of growth and cell

division to inhibition of ethylene production and senescence (Apelbaum *et al.*. 1981; Altman, 1982). Besides participating in the control of nucleic acid metabolism, protein synthesis and growth, polyamines are involved in plant response to environmental stress. An enhanced putrescine biosynthesis in plant tissues is a common feature of a wide range of stress conditions (Weinstein *et al.*, 1986; Di Tomaso *et al.*, 1989; Reggiani *et al.*, 1989; ; ShevyaKova *et al.*, 1994). Foliar application of putrescine on salt stressed rice plants increased shoot growth, grain yield, inhibited Na^+ and Cl^- uptake and accelerated the accumulation of K^+, Ca^{+2}, Mg^{2+} and proline (Krishnamurthy, 1991). Similarly, Prakash and Prathapasenan (1988), reported that putrescine when added together with NaCl effectively reduced the net accumulation of sodium and chloride ions in rice seeds and enhanced their water uptake. Polyamines have also been effective in maintaining the thylakoid membrane integrity of osmotically stressed oat leaves (Besford *et al.*, 1993).

Identification and quantitation of proteins has been used by many workers to provide a correlation between the altered expression of specific genes and changes in the environment. Several new proteins which are synthesized in response to an altered environment have been reported as stress proteins or shock proteins in plants (Stuart and Varner, 1980; Ericson and Alfinito, 1984). In response to NaCl also, proteins show an increased or decreased level of synthesis. Two new protein bands of 59Kd and 90Kd were detected in NaCl tolerant calluses of *Setaria italica* (Jia *et al.*, 1993), and NaCl tolerant cell line of millet (Lu and Jia, 1994). *De novo* induction of three new proteins (74 Kd,

28.5 Kd and 26.2 Kd) has been reported by Ramagopal(1986). Singh *et al.*(1985) have observed that there is involvement of a major 26 Kd polypeptide in the adaptation of the cells to NaCl.

Rice is an important staple food for two third of world population and accounts almost half of the daily caloric intake of people in Asian countries. Due to the various advantages of *in vitro* techniques, a large number of elite varieties have been developed through tissue and cell culture techniques. In the past most of the work on developing salt tolerant rice varieties involved direct selection i.e. screening for mutant and variant cell lines in culture that are able to grow on otherwise inhibitory levels of NaCl. But the sexual transmission of this character *in vivo* is not always observed in absence of the selection pressure. This inability is due to the fact that the cells in culture either develop salt exclusion mechanism or undergo minor epigenetic variations during the selection procedure. These characters are soon lost during the regeneration of plantlet. Thus to ascertain the presence of salt tolerance even in the regenerated plant, a different strategy has been adopted in the present work for developing stable salt tolerant cell lines of rice. In this work, the strategy adopted was isolating hydroxyproline (which is a toxic analogue of proline) resistant rice cell lines. The basic principle employed here is that resistance of a cell line to a toxic amino acid analogue is caused mostly by overproduction of the corresponding amino acid (Maliga, 1984). This strategy has proved successful in case of a number of other plants like tobacco (Widholm, 1976), potato (Van Swaaij *et al.*, 1986), barley (Kueh and Bright, 1981), carrot (Widholm, 1976), wheat (Tantau and Dorffling, 1991) etc.

Proline status of plant organs and cell cultures continues to be an active area of research in stress physiology (Steward and Larher, 1980; Aspinall and Paleg, 1981), as it serves a number of important functions in stressed cells. Elevated levels of proline are believed to protect plant tissues against stress by acting as N- storage compound, osmo-solute and hydrophobic protectant for enzymes and cellular structures (Stewart and Lee, 1974). Recently proline synthesis has been shown to be linked with oxidation of NADH to NAD in the mitochondria, which is hampered severely during salt stress and is one of the major causes of cell death during salt and mineral toxicity. (Alia and Pardha Saradhi, 1993; Pardha Saradhi *et al*, 1993). Proline effected salt tolerance has been reported in several crops and cell cultures (Pandey and Ganapathy, 1985; Kumar and Sharma, 1989). Thus it is indicated that proline overproducing cell line will be salt tolerant (Li 1990; Prakash and Sarin, 1993; ShevyaKova *et al.*, 1994; Vazquez-Flota and Loyola-Vargas, 1994).

The present study was therefore undertaken with the following objectives :

i) To understand the mechanism of salt tolerance in the tolerant (Bhoora rata-BR) and the susceptible (GR_{11}) varieties of rice (*Oryza sativa* L.).

ii) To isolate hydroxyproline resistant cell lines of rice.

iii) To understand the physiological basis of salt tolerance rendered by hydroxyproline.

iv) Develop a protocol for complete plantlet regeneration of hydroxyproline resistant calli.

The results of these studies are discussed in the light of relevant literature and are presented in this thesis.