Theor Appl Genet (1986) 71:757–760

In vitro characterization of salt stress effects and the selection of salt tolerant plants in rice (*Oryza sativa* L.)

P. Janardhan Reddy and K. Vaidyanath

Department of Genetics, Osmania University, Hyderabad 500 007 AP, India

Received August 4, 1985; Accepted September 12, 1985
Communicated by B. R. Murty

Summary. The response of plant cells to salt stress was studied on embryo derived calli of rice (*Oryza sativa* L.) in order to identify cellular phenotypes associated with the stress. The feasability of selecting salt tolerant callus and its subsequent regeneration to plants was also studied. Callus was grown on agar-solidified media containing 0%, 1% and 2% (w/v) NaCl for 24 days. Parameters such as fresh weight, dry weight, soluble protein and proline content were measured. The callus growth decreased markedly with increasing NaCl concentration in the medium. The proline content was enhanced several fold in salt stressed calli. A prolonged exposure of callus to the salt environment led to discolouration and arrested growth in the majority of the calli and only a small number of callus cells maintained healthy and stable growth. These variants were subcultured every three weeks for a period of four months onto medium containing 1% NaCl to identify tolerant lines. At the end of the third cell passage, the tolerant calli were transferred to regeneration medium to regenerate plants. The regeneration frequency in the salt-selected lines was enhanced when compared to unselected lines.

Key words: *Oryza sativa* – Rice – Callus – Regeneration – Salt tolerance

Introduction

Salinity, a serious problem affecting one third of all the irrigated land in the world (Mass and Hoffman 1977), impairs normal growth and limits the realization of yield potential of modern cultivars.

Though reclaimation procedures are advocated as a solution to the problem, such steps are often expensive and are seldom effective. Such a predicament logically leads to the idea of evolving salt tolerant varieties by manipulating the heritable variations present in the germplasm through plant breeding methods (Bhumbla et al. 1968; Ponnamperuma 1978). This conventional approach is not only cumbersome but also a time-consuming process. In recent times, in vitro techniques have proved to be useful in diverse areas of plant research and have been used successfully to develop variant cell lines from somatic populations (Dix and Street 1975; Nabors et al. 1975; Croughan et al. 1978).

In view of the economic importance and also the low salt tolerance shown by rice when compared to other cereals like wheat and barley, the present study was undertaken to select salt tolerant plants using the tissue culture technique. This report deals with the effect of NaCl on callus tissue and the identification and isolation of tolerant callus cell lines.

Materials and methods

Cultural conditions for callus induction

Dehulled seeds of Basmati rice (cv. 27,814) were surface sterilized for 10 min with 70% ethanol and for 1 min with 0.1% aqueous mercuric chloride solution. After several rinses with sterilized distilled water, the seed were germinated for 36 h at 24 ± 1 °C. Embryos were excised from each seed; five to six such embryos per tube were placed onto solidified agar medium containing Linsmaier and Skoog's medium (1965), supplemented with 2 mg/l of 2,4-dichlorophenoxy acetic acid (2,4-D). All cultures were kept under continuous white influorescent light and maintained for 3 weeks at 28 ± 2 °C to induce callus. Part of the callus was transferred to fresh medium and allowed to proliferate for 3 weeks under the same conditions. Two sets of experiments were conducted using the callus: one to investigate the effects of NaCl stress on callus growth and metabolism, and another to select salt tolerant lines and subsequently regenerate them.

Study of the effects of NaCl on rice callus

Proliferated callus was divided into small pieces, each weighing about 50 mg. These pieces (one per tube) were placed onto agar solidified LS media containing 0%, 1% and 2% NaCl in three replications. All culture tubes were maintained for 3 weeks. After 3 weeks, fresh and dry weights of the callus and content of soluble protein and proline was estimated; protein content was estimated following the procedure of Lowry et al. (1951) and the proline content, following the method of Bates et al. (1973).

Procedure for the selection of salt tolerant rice cultures

Four to five small pieces of callus per tube were exposed to agar-solidified nutrient medium (LS medium) containing 1% NaCl and were maintained at 28±2°C, under continuous illumination. The elevated tolerance of the callus to NaCl was identified visually based on normal growth of the callus despite salt stress. These lines were isolated and subcultured again under the same conditions in the presence of NaCl. Those callus lines that were consistantly salt tolerant upon repeated subculture on saline nutrient medium were used for the regeneration of plants. The isolated salt tolerant callus lines were subcultured on selective medium for three passages, each passage lasted three weeks.

Regeneration

The salt tolerant callus at the end of the third subculture was transferred to tubes containing regeneration medium (LS + 1 mg of IAA and 6 mg of kinetin/l) and kept at 28±2°C under continuous illumination with influorescent light.

Results

Callus initiation and growth under salt stress

Callus initiation was observed within six days after inoculation of the embryo explants onto LS medium with 2 mg of 2,4-D/l. The frequency of callus initiation was 84% in control calli.

Table 1 presents data on the growth of the callus and the attendant biochemical changes under varying concentrations of sodium chloride. Salt stress affected the growth of the callus: callus growth decreased as the concentration of sodium chloride increased. The reduction in fresh weight, expressed as percent of the control, was 67.92% with the 1% NaCl treatment and 56.8% with the 2% NaCl treatment. The variation in dry weight of the callus grown under varying concentrations of sodium chloride also showed a similar response: at 1% NaCl it was 72.34% and at 2% NaCl it was 53.19%.

The comparison of simple biochemical parameters such as soluble protein and proline content between control calli and calli grown under varying sodium chloride concentrations revealed that salt stress affects both these parameters differentially. The soluble protein content in control calli was 89 mg/g fresh weight, whereas it was 76.5 mg/g fresh weight at 1% NaCl and 71 mg/g fresh weight at 2% NaCl. The decline in the soluble protein content increased with an increase in the salt concentrations. The percentage decrease was 14% at 1% NaCl, and 21% at 2% NaCl.

Table 1. Growth and changes in the biochemical profile in the embryo calli of Basmati rice (cv. 27814) under salt (NaCl) stress

Parameter	Stress level (% w/v NaCL)		
	0	1%	2%
Fresh weight (mg)	212.0	144.0	120.0
Growth on fresh weight basis (expressed as % control)	100.0	67.0	56.0
Dry weight (mg)	47.0	34.0	25.0
Growth on dry weight basis (expressed as % control)	100.0	72.0	53.0
Soluble protein content (mg/g fresh weight)	89.0	76.5	71.0
Protein content (expressed as % control)	100.0	85.0	79.0
Proline content (mg/g fresh weight)	0.18	1.03	1.20
Proline accumulation (expressed as % control)	100.0	572.0	666.0

The proline content in stressed calli was different from the non-stressed control: it increased several fold in the stressed callus. The controls showed about 0.18 mg/g fresh weight of proline; at 1% NaCl proline content was 1.03 mg/g fresh weight, and at 2% NaCl it was 1.20 mg/g fresh weight. The increase in proline accumulation at 1% NaCl was 572%, and at 2% NaCl it was 666%, when expressed as percent of control.

Selection of salt tolerant callus lines

Out of 1,500 control cultures inoculated for callusing in the medium (LS+2,4-D, 2 mg/l), 1,260 showed callusing ability amounting to 84%. However, in the selection medium (LS+2,4-D, 2 mg/l+1% NaCl), only 192 of the 1,200 cultures inoculated callused (amounting to 16%). Over repeated subculturing in selective medium containing 1% NaCl, the growth of the callus was poor. Nevertheless, a few callus lines showed a healthy and stable growth throughout the three selective passages. The calli sensitive to NaCl stress (1%) showed discolouration and were predominantly brown whereas, the tolerant callus lines did not exhibit any sort of discolouration or browning.

The regeneration of salt tolerant plants

Regeneration of tolerant callus was accomplished by transferring the stable salt tolerant lines after three cell

passages through selective medium into regeneration media. From the 120 of both selected and unselected (control) calli cultures kept for regeneration, 74 were regenerated in controls whereas 87 of them were regenerated in lines selected for salt tolerance. The regeneration frequency expressed in percentage was 61% in the case of unselected lines (control) and 72% in the case of selected lines.

Discussion

Under non-stressed conditions calli grew faster when compared to the stressed ones. The efficiency of callus initiation was 84% in the culture medium without NaCl (control). On the other hand the callus exposed to NaCl showed symptoms of retardation in growth. Salt stress decreased fresh weight, dry weight and soluble protein content of the callus when compared to control (Table 1).

Smith and McComb (1981) also obtained similar results in callus grown under varying concentrations of NaCl. The exposure of callus to a saline environment may lead to water stress and specific ionic imbalances perhaps resulting in ion toxicity. Furthermore, cells grown under stress may have to spend more metabolic energy than those grown in the absence of stress (Croughan et al. 1981). This extra energy most probably is used up in regulating osmotic adjustment. The observed decline in freshweight, dry weight and soluble protein content (Table 1) in the NaCl environment may be due to the diversion of some quantum of energy from growth and metabolism. In addition, the decline also may be as a sequel to the increase in the maintenance cost of growing cells under stress.

The data on free proline content indicated that, with an increase in the concentration of NaCl there is a concomitant increase in the proline content (Table 1), thereby implying that proline accumulates as a response to stress. The rapid accumulation of proline under varying concentrations of NaCl may be one of the conspicuous metabolic changes often observed under stress. Stewart and Lee (1974) suggested that proline might act as a cytoplasmic osmoticum to counteract the effect of increased accumulation of salt ions in the vacuoles. The observed, several-fold increase in levels of proline in calli grown in the saline environment in the present study, when compared to controls, also indicated that proline may be a metabolic device to maintain osmotic balance under stressful situations.

The exposure of proliferated callus to medium containing 1% NaCl, permitted selection of callus lines plausibly having enhanced salt tolerance. During the first selective passage, the callus growth was 16 percent. On repeated culturing in the selection medium, few lines maintained healthy and stable growth throughout the three selective passages. Similar in vitro studies for selecting salt tolerant cell lines were successfully accomplished in *Nicotiana tabbaccum* (Nabors et al. 1975), *Nicotiana sylvestris, Capsicum annuam* (Dix and Street 1975), *Medicago sativa* (Croughan et al. 1978) and *Oryza sativa* (Rains et al. 1980).

The salt tolerance shown by selected cell lines in the present study may be due to a shift towards a halophytic nature (Croughan et al. 1978) in the case of cell lines selected for salt tolerance from cultured *Alfalfa* cells. While explaining the mechanism of salt tolerance in halophytes, Flowers et al. (1977) suggested that the tolerance may be due to their ability to accumulate certain ions into the cytoplasm. In addition, salt selected cell lines may produce internally organic acids and amino acids or accumulate ions from the external medium thereby acquiring a high internal concentration of osmotically active solutes (osmolytes), just sufficient enough to maintain water balance (Flowers et al. 1977; Greenway and Munns 1980). In other words, the salt resistant cell lines in the saline environment are capable of maintaining a favourable water relation to cope with potentially toxic ions by obtaining the requisite nutrients despite the predominance of other ions in the external media. Though the mechanism of salt tolerance in selected salt resistant callus isolated in the present investigation was not studied, it may be perceived that a similar mechanism of tolerance may be in operation.

The regeneration frequency was high in cell lines selected for salt tolerance (72%), when compared to unselected ones (61%). The reduced regeneration ability in the controls may be as a result of the increased somatic age, which in turn accentuates the occurrence of aneuploids among callus cells (Murashige and Nakano 1967) in tobacco. The observed increase in the regeneration of salt tolerant calli may be due to the fact that cells grown under stress have reduced cell division, which in turn reduces the frequency of the aneuploid cells, thus paving the way for high regeneration ability (Kazuhirosuenga et al. 1982). Considering the decline in regeneration in controls due to other factors, it is significant that selection for salt tolerance has no way affected the regeneration ability in salt tolerant callus lines.

The present study is an attempt to characterize the physiological and biochemical effects of salt stress through tissue culture methodology and to obtain well-defined phenotypes of salt tolerant genotypes under in vitro conditions. This will help the plant breeder to select and isolate new variants for the improvement of crop plants by manipulating the tissue culture technology, which may serve as an adjunct to conventional plant breeding methods.

Acknowledgements. The authors wish to acknowledge Dr. G. M. Reddy, Professor of Plant Genetics, Osmania University, for facilities, and the Department of Atomic Energy, Government of India, for financial assistance.

References

Bates LS, Waldrem RP, Teare ID (1973) Rapid determination of free proline for water stress studied. Plant Soil 39: 205–208

Bhumbla BR, Singh B, Singh NT (1968) Effect of salt on seed germination. Indian J Agron 13: 181–185

Croughan TP, Stavarek SJ, Rains DW (1981) In vitro development of salt resistance of plants. Environ Exp Bot 21: 317–324

Croughan TP, Stavarek SJ, Rains DW (1978) Selection of NaCl tolerant line of cultured Alfalfa cells. Crop Sci 18: 959–963

Dix PJ, Street HE (1975) Sodium chloride resistant cultured cell lines from *Nicotiana sylvestris* and *Capsicum annuam.* Plant Sci Lett 5:231–237

Flowers IJ, Troke PF, Yeo AR (1977) The mechanism of salt tolerance in halophytes. Annu Rev Plant Physiol 28: 89–121

Greenway H, Munns R (1980) Mechanisms of salt tolerance in non-halophytes. Annu Rev Plant Physiol 31:149–190

Hanson AD, Nelson CE, Pederson AR, Everson EH (1979) Capacity for proline accumulation during water stress in barley and its implications for breeding for drought resistance. Crop Sci 19:489–493

Kazuhirosuenga Editha M, Abrigo Shouichi Yoshida (1982) Seed derived callus culture for selecting salt tolerant rices. IRRS 79:1–11

Linsmaier EM, Skoog F (1965) Organic growth factor requirement for tobacco tissue cultures. Physiol Plant 15:473–497

Lowery OH, Rose Brought NO, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275

Mass EV, Hoffman GJ (1977) Crop salt tolerance evaluation of existing data. In: Drengne HE (ed) Managing saline water for irrigation. Texas Tech University Press, Lubback, Texas, pp 16–20

Murashige T, Nakano R (1967) Chromosome complement as a determinant of the morphogenic potential of tobacco cells. Am J Bot 54:963–970

Nabors MW, Daniels A, Nadolny L, Brown C (1975) Sodium chloride tolerant lines of tobacco cells. Plant Sci Lett 4:155

Nabors MW, Gibbs SE, Bernstein CS, Meis ME (1980) NaCl tolerant tobacco plants from cultural cells. Z Pflanzenphysiol 97:13–17

Ponnamperuma FN (1977) Breeding Crop Plants to tolerate soil stresses. In: Scowcroft WR (ed) Somatic cell genetic and plant improvement. Advances in agronomy. Academic Press, New York, pp 73–97

Rains DW (1972) Salt transport by plants in relation to salinity. Annu Rev Plant Physiol 23:367–388

Rains DW, Croughan TP, Stavarek SJ (1980) Selection of salt tolerant plants using tissue culture. In: Rains DW, Ualentine RC, Hollaender A (eds) Genetic engineering of osmolegulation: impact on plant productivity for food, chemicals and energy. Plenum Press, New York, pp 279–292

Rains DW, Epstein EC (1967) Preferential absorption of potassium by leaf tissue of the mangrove, *Avicennia marina,* an aspect of halophytic competence in coping with salt. Aust J Biol Sci 20:847–858

Smith MK, McComb JA (1981) Effect of NaCl on the growth of whole callus cultures. Aust J Plant Physiol 8:267–275

Stewart GR, Lee JA (1974) The role of proline accumulation in halophytes. Plant 120:279–281