Materials and Methods

MATERIALS AND METHODS

Source of Seeds

Certified seeds of two varieties of rice (*Oryza sativa L.*), namely Bhoora rata (BR-salt tolerant) and GR_{11} (salt susceptible) were obtained from Main Rice Research Station, Gujarat Agricultural University, Nawagam and are used in present studies.

Chemicals

All chemicals used in experiments were of high purity grade. They were obtained from British Drug House (Anala R grade), Loba, Qualigens, Sarabhai, Merck (Guaranteed Reagents) and Sigma Chemical Company (U.S.A.).

Culture Vessels

All glasswares used in the present study were of Corning brand. Depending upon the type of culture, Erlenmeyer conical flasks of 150 ml capacity and test tubes of 25 X 150 mm capacity were used as culture vessels. Before use, all glassware used for the preparation of media or other analysis were thoroughly cleaned as per standard procedures.

Preparation of Media

For the induction and maintenance of callus, Linsmaeir and Skoog's medium (L.S.medium, 1965)was employed. The composition of basal L.S. medium is given in Table 1.

The basic media were prepared from concentrated stock solutions. Other

supplements to the basal medium, like sucrose and 2,4-Dichlorophenoxyacetic acid (2,4-D) were also added. The pH of the medium was adjusted to 5.8 using either 0.1 N HCl or 0.1N NaOH and the volume was made up to the required level using double glass distilled water. Agar (Qualigens) was added to the medium to have a final concentration of 0.8% (w/v). The medium was then heated to dissolve the agar.

The prepared medium was then distributed into tubes (20 ml aliquots) or into flasks (30 ml aliquots) for autoclaving. The culture vessels were closed with non-absorbent cotton plugs. Cotton plugs were covered with paper to protect them from condensed water during autoclaving. Scalpels, forceps, spatulas, Petri dishes etc. were also wrapped with paper for sterilization. Sterilization was carried out by autoclaving at a pressure of 15 psi (121°C) for 20 minutes.

Culture Technique

All inoculations and manipulations involving sterile cultures or media were carried out in a laminar flow cabinet (Klenzaids, India). The instruments used were sterilized by flaming them with absolute alcohol on a gas burner inside the cabinet during inoculations.

Surface Sterilization of Seeds

Sterilization of the seeds was carried out by a three step procedure :

i) Washing intact seeds with 95% v/v aqueous ethanol for 2-3 minutes.

ii) These seeds were then dehusked and washed with soap and distilled water.

iii) Seeds were then sterilized with 0.1% w/v of $HgCl_2$ for two minutes and

washed three times thoroughly with sterile double distilled water. The last step was carried out in the laminar hood.

Inoculation Procedure

The sterilized seeds were then inoculated on sterile 0.8% agar based L.S.medium. The L.S. medium was supplemented with 11.31 μM, 2,4-D and 2% sucrose for callus induction.

Initiation and Maintenance of Callus Culture

Culture Conditions

After inoculation, the cultures were incubated for callus initiation at 25$\pm$2°C under 16 hour photoperiod by day light fluorescent (1200 lux intensity) tubes (Philips, India) .

Callus Induction and Maintenance

Proliferation of the callus took place at the root shoot juncture and at the base of the shoot. This callus was maintained on the same medium by periodic biweekly subculture.

Isolation of Hydroxyproline Resistant Cell Lines

The hydroxyproline resistant cell lines were isolated by culturing the four week old calli of both varieties of rice on L.S. medium containing 0.1,1,10 and 20 mM concentration of hydroxyproline. After fifteen days of incubation, the increase in the fresh and dry weights of the callus were recorded. It was observed that under 10 mM hydroxyproline (LD_{50} concentration), the increase in weight was half of the maximum weight of control (data not presented). This experiment was carried out with six replicates and repeated thrice. The resistant

calli produced at LD_{50} concentration were regularly subcultured on the same medium containing 10 mM hydroxyproline at two weeks interval.

In all subsequent experiments two week old calli (of both rice varieties) cultured on L.S. medium containing 0 and 10 mM of hydroxyproline were used and observations were made at the end of 0,2,4 and 6 weeks.

Growth Measurement

Known weight of the callus was cultured on L.S. medium containing 0 and 10 mM hydroxyproline and the increase in its dry weight was recorded at 0,2,4 and 6 weeks interval.

Extraction of Na^+, K^+, Mg^{2+}, Ca^{2+} and Cl^- ions

The procedure followed by Prakash and Prathapasenan (1988), was employed for the extraction of Na^+, K^+, Mg^{2+}, Ca^{2+} and Cl^- ions. Known weight of dry callus was extracted thrice with boiling deionised water and the supernatant was collected by centrifuging the suspension for 10 minutes at 6,000 X g. The residue was then extracted with 30% (v/v) nitric acid for 1 hour at 90°C. After cooling, the supernatant was collected by centrifuging at 6,000 X g for 10 minutes. The residue was re-extracted thrice with 30% nitric acid. All supernatants were pooled together and made upto a known volume.

Estimation of Na^+, K^+, Mg^{2+}, Ca^{2+} and Cl^- ions

Na^+ and K^+ ions were estimated flame photometrically (Systronics). Chloride was determined by titrating with silver nitrate using ferric ammonium sulphate as an indicator. The quantity of silver nitrate required for colour development was

recorded and chloride content of the solution was calculated using the following formula/equation :

Cl^- in grams/lt = [V X 35.45 X N]/S

where V = ml of silver nitrate consumed, N=normality of silver nitrate and S=ml ofsample (extract) assayed. Mg^{2+} amd Ca^{2+} ions were estimated using Atomic Absorption Spectrophotometer (Perkin-Elmer).

Extraction and Estimation of Total Protein

Known amount of fresh callus was homogenized in 5 ml of 0.3 N KOH and incubated for 18 hours at 37°C. The supernatant was collected by centrifugation of the suspension at 10,000 X g for 15 minutes and the residue washed thrice with 0.3 N KOH. The supernatants were pooled and the protein present was precipitated with 12% trichloroacetic acid. Precipitated protein was recovered by centrifuging at 10,000 X g for 25 minutes, and redissolved in 0.3 N KOH. An aliquot of this extract was used for the estimation of protein using Bradford's microassay (1976). Bovine serum albumin was used as standard.

Extraction and Estimation of Proline

Proline was extracted and estimated according to the method described by Bates *et al.* (1973).

Known weight of the callus was homogenized in 4 ml of 3% (w/v) aqueous sulfosalicylic acid and the homogenate was filtered through Whatman No.2 filter paper. 2 ml of the filtrate was reacted with 2 ml of acid ninhydrin (625 mg ninhydrin in 15 ml glacial acetic acid and 10 ml of 6M orthophosphoric acid)

and 2 ml glacial acetic acid in a test tube at 100°C for one hour and the reaction was terminated by keeping the tubes in icebath. The reaction mixture was extracted with 6 ml toluene. The chromophore containing toluene was aspirated from the aqueous phase and warmed at room temperature. The absorbance was read at 520 nm using toluene as blank. The proline concentration of the extract was determined from a standard curve of proline prepared according to the above procedure.

Preparation of Enzyme Extract for Different Enzyme Assays

A known weight of callus was homogenized in cold 0.2 M borate buffer (pH 8.6), along with a pinch of glass powder, for 5 minutes using a chilled mortar and pestle. The homogenate was centrifuged at 10,000 Xg for 10 minutes at 0°C. Chilled acetone at a ratio of 1:2 (v/v) at 0°C was added to the extract to precipitate the protein present. The precipitated protein was sedimented by centrifugation at 15,000 Xg for 15 minutes at 2°C and was dissolved in different buffers for various enzyme assays.

Total Amylase Activity

The method of Bernfeld (1955) was employed for determining the total amylase activity. The precipitated protein was dissolved in 0.05 M acetate buffer, pH 4.5. The assay system (2.0 ml) consisted of 0.5 ml of 0.1 M acetate buffer, pH 4.5; 0.5 ml of 1% soluble starch and 1.0 ml enzyme. The reaction was carried out for 30 minutes at 30 ± 1°C, and the amount of maltose released was estimated using 3,5-dinitrosalicylic acid. One enzyme unit is defined as the amount of enzyme required to liberate 100 μgs of maltose per 30 minutes

under the assay conditions and the activity is expressed as units per mg protein.

Cellulase Assay

For cellulase assay, the precipitated protein was dissolved in 0.05 M acetate buffer, pH 5.0. The method followed by Trivedi and Rao (1979), was employed with certain changes. The assay system contained 1.0 ml of 0.05 M acetate buffer (pH 5.0), 0.5 ml of enzyme and 0.5 ml of 1% carboxymethyl cellulose (CMC, Sigma), prepared in 0.05 M acetate buffer (pH 5.0). The reaction mixture was incubated at 37°C for 30 minutes. The reaction was terminated by heating the tubes in a boiling water bath for 5 minutes and an aliquot of the reaction mixture was used to determine the reducing sugars released according to the method of Somogyi (1952). One enzyme unit is defined as the amount of enzyme required to release 100 μg of reducing sugars per 30 minutes under the assay condition and the activity is expressed as units per mg protein.

Acid Invertase Assay

The activity of acid invertase was measured after dissolving the precipitated protein in 0.05 M acetate buffer, pH 4.6. Estimation of the reducing sugars produced in the assay system after 30 minutes of incubation was done. The assay system (2.0 ml) contained 1.0 ml of 0.1 M acetate buffer (pH 4.6), 0.5 ml of 2% sucrose and 0.5 ml enzyme extract. The reaction mixture was incubated for 30 minutes at 30 $\pm$ 1°C and the reaction was terminated by keeping the tubes in a boiling water bath for 10 minutes. An aliquot from the reaction mixture was used for the estimation of reducing sugars according to the

procedure of Somogyi (1952). One enzyme unit is defined as the amount of enzyme required to produce 100 μg of glucose per 30 minutes under the assay condition and the enzyme activity is expressed as units per mg protein.

IAA Oxidase Assay

IAA oxidase activity was determined by the method of Gordon and Weber (1951) after dissolving the protein pellet in 0.02 M phosphate buffer, pH 6.1. The reaction mixture of 4.0 ml consisting of 1.0 ml enzyme extract, 0.5 ml of 0.1 mM $MnCl_2$, 0.5 ml of 0.1 mM 2,4-dichlorophenol, 1 ml of 0.2 M phosphate buffer, pH 6.1, 1 ml of 0.1 mM Indole acetic acid (IAA), was incubated for 30 minutes at 30 $\pm$ 1°C in dark. After incubation 2.0 ml of the reaction mixture was added to 4.0 ml modified reagent and the colour was allowed to develop for 25 minutes. The absorbance of the solution was measured at 530 nm. One enzyme unit is defined as the amount of enzyme required to oxidise 50 μg of IAA per 30 minutes under the assay condition and the enzyme activity is expressed as units per mg protein.

Assay of Proline Oxidase

The protein pellet was dissolved in 0.1 M Tris-HCl buffer, pH 8.0 for the assay of proline oxidase. The method of Huang and Cavalieri (1979), was followed. The 5 ml reaction mixture consisted of 50 mM Tris-HCl (pH 8.0), 2.5 mM $MgCl_2$, 0.5 mM FAD, 1 mM Phenazine methosulfate, 0.06 mM dichlorophenolindophenol, 5 mM proline and 1.0 ml of enzyme extract. The reaction was monitored at 600 nm at 30 $\pm$ 1°C using proline to initiate the reaction. One unit of enzyme is defined as the amount of enzyme which cause a

change in optical density of 0.01 per minute at 600 nm under the assay condition and enzyme activity is expressed as units per mg protein.

Extraction and Estimation of Polyamines

The method of Flores and Galston (1982) was employed for the extraction and quantification of polyamines. Tissues were extracted in 5% cold perchloric acid ($HClO_4$) at a ratio of about 200 mg/ml $HClO_4$. After extraction for 1 hour in an ice bath, samples were pelleted at 15,000 Xg for 30 minutes, and the supernatant phasecontaining the `free' polyamine fraction, was stored frozen in plastic vials.

Standards and plant extracts were benzoylated according to Redmond and Tseng(1979). One ml of 2 N NaOH was mixed with 1 ml of $HClO_4$ extract. After addition of 20 μl benzoyl chloride, vortexing for 10 seconds and incubation for 20 minutes at room temperature, 2 ml of saturated NaCl was added. Benzoyl polyamines were extracted in 2 ml of diethyl ether. After centrifugation at 15,000 Xg for 5 minutes, 1 ml of the ether phase was collected, evaporated to dryness, and redissolved in 500 ml of methanol. Standards were also treated in a similar way. High Performance Liquid Chromatography analysis was done with LKB liquid chromatograph. The solvent system consisted of methanol : water (64% v/v), run isocratically at a flow rate of 1 ml/min. The benzoylated extracts were eluted at room temperature through a 4.6 x 250 mm, 5 μm particle size reverse phase (C_{18}) column and detected at 254 nm.

Assessment of Hydroxyproline Resistant Calli for their Tolerance to Salinity

The hydroxyproline resistant calli of both varieties of rice were transferred to 0,50,100 and 200 mM concentrations of NaCl. After two weeks of incubation, the increase in dry weight of the callus was recorded. It was observed that under 200 mM NaCl (LD_{50} concentration) the increase in weight was half of the maximum weight of control (data not presented). This experiment was repeated thrice with five replicates. To assess their tolerance to salinity, the hydroxyproline resistant calli of both rice varieties were transferred to L.S. medium containing 200 mM NaCl. The hydroxyproline exposed calli were grown on two different media :

a) L.S.medium containing 200 mM NaCl

b) L.S.medium containing 200 mM NaCl and 10 mM hydroxyproline

Regeneration of Hydroxyproline Resistant Cell Lines of Rice

Murashige and Skoog's medium (MS medium, 1962) supplemented with 3% sucrose, 13.32 μM Benzyladenine (BA) and 2.46 μM Indole butyric acid (IBA) was employed for regeneration of hydroxyproline resistant cell lines of both rice varieties. The composition of basal MS medium is given in Table 2. The pH of the medium was adjusted to 5.8. Agar was added to the medium to get a final concentration of 0.8% (w/v). The medium was heated to dissolve the agar. The prepared medium was then distributed into culture tubes (20 ml aliquot) for autoclaving.

Callus cultures of both rice cultivars (approx. 200 mg) grown on L.S. medium containing 0 and 10 mM concentration of hydroxyproline were transferred to the

regeneration medium containing 200 mM NaCl. Cultures were maintained at 25 ± 2°C under 16 hour photoperiod (cool white fluorescent tubes, 1200 lux intensity). After 3-4 weeks of growth on the regeneration medium, the plantlets were transferred to above fresh medium for further growth.

Table 1

Linsmaier and Skoog's (1965) medium

Concentration in 1 litre of complete medium as mg of hydrated salts.

Ammonium Nitrate	NH_4NO_3	1650.00
Potassium Nitrate	KNO_3	1900.00
Calcium Chloride	$CaCl_2 2H_2O$	370.00
Magnesium Sulpate	$MgSO_4.7H_2O$	440.00
Potassium dihydrogen phosphate	KH_2PO_4	170.00
Na_2EDTA		37.26
$FeSO_4.7H_2O$		27.8
Microelements and Vitamins		
Boric Acid	H_3BO_3	6.20
Potassium Iodide	KI	0.83
Sodium Molybdate	$Na_2MoO_4.2H_2O$	0.25
Cobalt chloride	$CoCl_2.6H_2O$	0.025
Manganese Sulphate	$MnSO_4H_2O$	16.9
Zinc Sulphate	$ZnSO_4.7H_2O$	8.6
Copper Sulphate	$CuSO_4.5H_2O$	0.025
Thiamine hydrochloride		2.0
Supplements		
Sucrose		20.0 gms
Myo-inositol		100.0 mg

Table 2

Murashige and Skoog's (1962) medium

Concentration in 1 litre of complete medium as mg of hydrated salts.

Ammonium Nitrate	NH_4NO_3	1650.00
Potassium Nitrate	KNO_3	1900.00
Calcium Chloride	$CaCl_2.2H_2O$	440.00
Magnesium Sulphate	$MgSO_4.7H_2O$	370.00
Potassium dihydrogen phosphate	KH_2PO_4	170.00
Microelements and Vitamins		
Boric Acid	H_3BO_3	6.20
Potassium Iodide	KI	0.83
Sodium Molybdate	$Na_2MoO_4.2H_2O$	0.25
Cobalt chloride	$CoCl_2.6H_2O$	0.025
Manganese Sulphate	$MnSO_4.4H_2O$	22.3
Zinc Sulphate	$ZnSO_4.7H_2O$	8.6
Copper Sulphate	$CuSO_4.5H_2O$	0.025
Ferric Sulphate	$FeSO_4.7H_20$	27.8
Thiamine hydrochloride		
Na_2EDTA		37.26
Thiamine hydrochloride		1.0
Pyridoxine hydrochloride		1.0
Glycine		4.0
Nicotinic acid		1.0
Supplements		
Sucrose		30.0 gm
Myo-inositol		100.0 mg