MATERIALS AND METHODS

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Source of Seeds

Certified seeds of rice (*Oryza sativa* L. var. CSR-10) obtained from Main Rice Research Station, Gujarat Agricultural University, Nawagam, Gujarat were used in the present research work.

Chemicals

All chemicals used in the preparation of culture media and also for various biochemical studies were of analytical grade. They were obtained from British Drug House (Analar Grade), Loba, Qualigens, Sarabhai, E. Merck (Guaranteed Reagents). Auxins, cytokinins and vitamins were obtained from Sigma Chemical Company (U.S.A.)

Glassware

All the glasswares specially culture flasks, test tubes, petridishes etc. used in the present studies were either Borosil or Corning make. Erlenmeyer flasks with 150 ml capacity or 50 ml test tubes (150 x 26mm) were used as culture vessel.

Glasswares for the cultural studies and chemical analysis were cleaned with chromic acid -sulphuric acid mixture (Alexopoulos and Beneke, 1955) prepared by dissolving 100 gm of potasium dichromate in 500 ml of concentrated sulphuric acid and diluted to 2:1 with water. Prolonged rinsing with diluted teepol (a mild detergent) was done to remove traces of chromic acid followed by 4-5 rinses with tap water. The glassware was finally rinsed with distilled water throughly and kept in hot air oven at 60°C for 24 hours.

Culture media preparation

Basal medium formulated by Murashige and Skoog (MS, 1962) and Linsmaeir and Skoog (LS, 1965) were used in the present studies for the initiation

of callus, maintenance, induction of somatic embryogenesis and plantlet regeneration. The chemical composition of both media is given in Table 1 & 2.

Table 1: Murashige and Skoog medium (1962)

1.1 Macroelement concentrations

Chemical	Original concentration (mgl^{-1})	Concentration of stock solution (mgl^{-1})	Volume of stock per litre of medium
NH_4NO_3	1650	33,000	
KNO_3	1900	38,000	
$CaCl_2.2H_2O$	440	8,800	50 ml
$MgSO_4.7H_2O$	370	7,400	
KH_2PO_4	170	3,400	

Table 1 : Murashige and Skoog (1962)

1.2 Microelement concentrations

Chemical	Original concentration (mgl^{-1})	Concentration of stock solution (mgl^{-1})	Volume of stock per litre medium
KI	0.83	166	
H_3BO_3	6.20	1240	
$MnSO_4.4H_2O$	22.3	4460	
$ZnSO_4.7H_2O$	8.60	1720	
$Na_2MoO_4.2H_2O$	0.25	50	5 ml
$CuSO_4.7H_2O$	0.025	5.0	
$CoCl_2.6H_2O$	0.025	5.0	

Table 1: Murashige and Skoog medium (1962)

1.3 Iron Source

Chemical	Original concentration (mgl^{-1})	Concentration of stock solution (mgl^{-1})	Volume of stock per litre medium
$FeSO_4.7H_2O$	27.8	5560	
$Na_2EDTA.2H_2O$*	37.3	7460	5 ml

*The $FeSO_4.7H_2O$ is dissolved in approximately 200 ml of dist. H_2O. The Na_2.EDTA is dissolved in approximately 200 ml of dist. H_2O, seperately heated and mixed (under continuous stirring) with $FeSO_4$ until it dissolves completely. The whole mixture was boiled for five minutes. After cooling, the volume was adjusted to 1000 ml. Heating and stirring resulted in more stable FeEDTA complex.

Table 1 : Murashige and Skoog medium (1962)

1.4 Organic components

Chemical	Original concentration (mgl^{-1})	Concentration of stock solution (mgl^{-1})	Volume of stock per litre medium
Myo-inositol	100	20,000	
Nicotinic acid	0.5	100	
Pyridoxine-HCl	0.5	100	5 ml
Thiamine-HCl	0.1	10	
Glycene	2.0	400	

Table 2 : Linsmaeir and Skoog (1965)

2.1 Macroelement concentration

Chemical	Original concentration (mgl^{-1})	Concentration of stock solution (mgl^{-1})	Volume of stock per litre medium
NH_4NO_3	1650	33,000	
KNO_3	1900	38,000	
$CaCl_2.2H_2O$	440	88,000	50 ml
$MgSO_4.7H_2O$	370	74,000	
KH_2PO_4	170	3,400	

Table 2: Linsmaeir and Skoog medium (1965)

2.2 Microelement concentrations

Chemical	Original concentration (mgl^{-1})	Concentration of stock solution (mgl^{-1})	Volume of stock per litre medium
KI	0.83	166	
H_3BO_3	6.20	1240	
$MnSO_4.4H_2O$	22.3	4460	
$ZnSO_4.7H_2O$	8.60	1720	5 ml
$Na_2MoO_4.2H_2O$	0.25	50	
$CuSO_4.5H_2O$	0.025	5.0	
$CoCl_2.6H_2O$	0.025	5.0	

Table 2 : Linsmaeir and Skoog (1965) medium

2.3 Iron source

Chemical	Original concentration (mgl^{-1})	Concentration of stock solution (mgl^{-1})	Volume of stock per litre medium
$FeSO_4.7H_2O$	27.8	5560	5 ml
$Na_2EDTA.2H_2O$	37.3	7460	

Table 2 : Linsmaeir and Skoog medium (1965)

2.4 Organic components

Chemical	Original concentration (mgl^{-1})	Concentration of stock solution (mgl^{-1})	Volume of stock per litre medium
Myo-inositol	100	20,000	
Thiamine-HCl	2.0	400	5 ml

In order to reduce the time taken to weigh out each of the chemicals required for medium preparation every time, concentrated stock solutions were prepared and stored either at 5°C in refrigerator (stock solutions of inorganic solvents) or at -4°C in deep freezer (that of vitamins and hormones)

Basal medium was prepared by mixing the stock solution in a precise manner and the supplements like sucrose, 2,4-Dichlorophenoxy acetic acid (2,4-D) etc. were added prior to final volume adjustment. The pH of the medium was adjusted to 5.8 using either 0.1N HCl or 0.1N KOH and the volume is made up using distilled water. To gel the medium agar (0.8% w/v, Qualigens, Bombay) was added to the medium and was dissolved by gentle heating with constant stirring.

The prepared medium was then dispensed into culture tubes (20 ml aliquotes) or into flasks (30 ml aliquotes) for autoclaving. Liquid media were distributed directly in tubes 10 ml or in flasks 30 ml. The culture vessels were plugged and then covered with paper to protect the plugs from getting wet with condensed water during autoclaving. Scalpels, forceps, spatulas, petridishes etc. were wrapped in paper properly before sterilization. Sterilization was carried out by autoclaving at a pressure of 15 psi (121°C) for 20 minutes.

Aseptic Conditions

All tissue culture manipulations like surface sterilization, inoculations, subcultures etc. were carried out in a sterile laminar flow hood cabinet (Klenzaids, India, or ADC Bombay). Prior to inoculation the laminar flow hood bench was swabbed with 70% ethyl alcohol. After wiping with alcohol the culture vessels were placed on bench under UV light (λ 2357 A°) for proper sterilization. The materials used for inoculations and subculture (scalpels and forceps) were sterilized by flaming them with absolute alcohol inside the inoculation chamber during inoculations.

Preparation of Explant

a) Surface sterilization of seeds

In order to avoid bacterial or fungal growth the explants were surface sterilized before they were used for further cultural studies. The intact seeds were washed with 95% aqueous ethanol for 2-3 minutes. These seeds were then dehusked and washed with teepol followed by 4-5 rinses with water. The dehusked seeds were then kept under the running tap water for 20-30 minutes. Further treatment was carried out under sterile conditions under the laminar flow hood. Seeds were then sterilized with 0.1% w/v of mercuric chloride ($HgCl_2$) for

2-3 minutes and were washed many times thoroughly with sterile double distilled water in order to remove the traces of mercuric chloride.

Inoculation Procedures

The sterilized seeds were then inoculated on sterile 0.8% (w/v) agar based LS medium. The LS medium supplemented with different combinations of 2,4-D and 2% (w/v) sucrose was used for the induction of callus.

Initiation and Maintenance of Callus cultures

Incubation

After inoculation the culture flasks or tubes were incubated for callus initiation at 25 ± 2°C under 16 hour photoperiod by day light fluorescent (1200 lux intensity) tubes (Phillips, India).

Callus induction and Maintenance

The callus induced was maintained on the same medium by regular subculturing. These stock calli tissues served as the inoculum for further experiments.

Isolation of NaCl Resistant cell lines

The NaCl resistant cell lines were isolated by culturing four week old calli following a strategy of direct adaptation. The callus was cultured on L.S medium containing different concentrations of NaCl (0-300mM). After two weeks of incubation, the increase in fresh and dry weights of the callus was recorded and the LD_{50} concentration was determined. The resistant cell lines produced at LD_{50} concentration of NaCl were regularly subcultured on the same medium containing LD_{50} concentration of NaCl.

Regeneration studies

The selected cell lines were inoculated on MS medium with different combination of growth regulators (IAA, Indole-3-acetic acid; NAA, 1-Naphthalene acetic acid; KN-Kinetin) under the same selection pressure. Salt adapted callus tissues were transferred to regeneration medium containing same concentration of salt. The regenerants were then transferred to pots for acclimatization and further growth.

Initiation and Maintenance of Suspension cultures

a) Induction of friable embryogenic callus

Loose and friable callus was obtained by culturing a known volume of callus in either LS basal medium or LS medium supplemented with low level (0.05 mg/l) of 2,4-D. These calli were repeatedly subcultured on the same medium and were used in further studies.

b) Establishment of cell suspensions

Friable embryogenic callus of about 250 mg of fresh weight was used for establishing cell suspensions. The well established suspensions were maintained on gyratory shaker (120 rpm) in the culture room at 25 ± 2°C with 16 hour photoperiod. These cultures were maintained by weekly subculture.

c) Establishment of Embryogenic cell suspensions

One month old friable callus was allowed to grow on LS medium containing LD_{50} concentration of NaCl. After 2-3 subcultures in the same medium they were directly transferred to embryogenic medium. A known amount (250 mg of NaCl adapted) callus was transferred to Erlynmeyer (150 ml) flasks containing LS basal medium for establishing suspension cultures. After 8-10 days the suspensions were composed of cells and cell clumps. This was filtered through a

stainless steel mesh (4.8 x 10^4 μm^2) and 5 ml of this filterate was transferred to 25 ml of embryogenic medium to get a total of 30 ml. The embryogenic response of these cultures was tested under varying concentrations of different hormones.

Experiments were conducted to reduce the problem of rhizogenesis in the induced embryos. Different concentrations of activated charcoal (0-8%) and NaCl (0-250 mM) were also tried in order to check rhizogenesis especially in embryoids formed from non-adapted cells.

Maturation was induced in embryoids by incorporating sucrose (0-6%, Poly Ethylene Glycol (PEG -6000, 0-6%) and mannitol (0-100 mM) in the medium.

Germination of embryoids was tried on filter paper in liquid medium as well as on solid medium.

In order to improve the efficiency of somatic embryogenesis and germination different amino acids viz. proline (0-32 mM) and hydroxyproline (0-32 mM) as well as polyamin viz. putrescine (0-0.8 mM) and an inhibitor of polyamine MGBG (methyl-glyoxal-bis guanyl hydrazone) (0-1mM) were also tried.

Encapsulation of embryoids

The small clusters of embryoids were mixed with 3% sodium alginate in half strength LS basal medium with 2% sucrose. The embryoids alongwith the alginate solution were dropped into 60 mM $CaCl_2$ solution with the help of a sterile pipette. The encapsulated embryoids were kept in the same solution for half an hour and later they were washed with sterile distilled water. To study the effect of storage on germination, the encapsulated embryoids were stored at different temperatures (6°C, 4°C, 2°C) on moist filter paper in sterile petri dishes, and covered with aluminium foil for 10-30 days.

BIOCHEMICAL ANALYSIS

a) Determination of total protein

Known amount of fresh callus (harvested from 0, 10, 20, 30 and 40 days) was homogenised in 5 ml of 0.3N KOH and incubated for 18 hours at 37°C. The supernatant was collected by centrifugation of the suspension at 10,000Xg for 15 minutes and the residue washed thrice with 0.3N KOH. The supernatants were pooled and the protein present was precipitated with 12% trichloroacetic acid. Precipitated protein was recovered by centrifuging at 10,000 Xg for 25 minutes, and was redissolved in 0.3N 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.

b) Determination of proline content

Extraction and estimation proline was done according to Bates *et.al.* (1973). A known amount of the callus was homogenised in 4 ml of 3% (w/v) aqueous sulfo salycilic acid and the homogenate was filtered through Whatman No. 2 filter paper. 2 ml of the filterate was allowed to react 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 of glacial acetic acid in a test tube. The test tubes are then incubated at 100°C for one hour and the reaction was terminated by keeping the tubes in ice bath. The reaction mixture was extracted with 6 ml of toluene. The chromophore containing toluene was aspirated from the aqueous phase, warmed to room temperature and the absorbence was read at 520 nm using toluene as a blank.

c) Determination of total amylase

A known weight of callus was homogenised in cold 0.2 M borate buffer (pH 8.6), along with a pinch of glass powder, for five minutes using a chilled mortar and pestle. The homogenate was centrifuged at 10,000 x g for ten 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 x g for 15 minutes at 2°C and was dissolved in respective buffers for the enzyme assays.

The total amylase assay was carried out according to the method of Bernfeld (1955). The precipitated protein was dissolved in 0.05 M acetate buffer, pH 4.5. The assay system of 2 ml consisted 0.5 ml of 0.1 M acetate buffer, pH 4.5, 0.5 ml of 1% starch and 1.0 ml of enzyme extract. The reaction was carried out for 30 minutes at 30 ± 1°C, and the amount of maltose released was estimated using 3,5-dinitrosalicyclic acid.

One enzyme unit is defined as the amount of enzyme that will release 100 µgs of maltose per 30 minutes under the assay conditions and the activity is expressed as units per mg protein.

d) Determination of IAA Oxidase

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 of enzyme extract, 0.5 ml of 0.1 M $MnCl_2$, 0.5ml of 2,4-dichlorophenol, 1 ml of 0.2 mM phosphate buffer, pH 6.1, 1.0 ml of 0.1 mM Indole acetic acid (IAA), was incubated for 30 minutes at 30 ± 1°C in dark. After incubation 2.0 ml of the reaction mixture was added to 4.0 ml of

modified Salkowski 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.

Statistical analysis

All the experiments were repeated thrice with four replicates in each experiment. Values are represented as mean ± standard error (S.E. of the replicates). The data were analysed by one way ANOVA in the case of biochemical studies and other experiments and the significant variations among the means were analysed by Duncan's multiple range test (Duncan 1952). All the computations and analyses were carried out on computer using 'SPSS for windows 6.0' statistical package.