MATERIALS AND METHODS

Source of seeds

Certified seeds of Oryza sativa L. var GR-3 were obtained from the State Department of Agriculture, Gujarat, India.

Method of cultivation

Plants were raised in pots, lined with plastic sheets, containing 7.0 kg. garden soil (three volume waste-land soil and one volume compost) under wire house conditions. Seeds, 20 per pot, were sown at a depth of 1 cm and were allowed to germinate. When seedlings were a week old they are thinned to 10 per pot. Pots were divided into six groups for different treatments. On day 21, two groups of plants were sprayed with putrescine solution, 10^{-5} M (the concentration which gave maximum growth stimulation in the preliminary studies - Table 1) in 0.02% (v/v) Tween 20 and two groups were sprayed with 10 ppm gibberellic acid (GA_3) in 0.02% (v/v) Tween 20, the concentration which gave maximum growth stimulation in an earlier study in the same laboratory (Acharya, 1983), to the point of run off prior to the imposition of salt treatment. Other two groups (control plants) received sprays of distilled water containing 0.02% Tween 20. Salt treatment (EC 12 dS/m, the salinity level which inhibited the dry matter production by about 50%) (Acharya, 1983) was imposed by

supplying NaCl through the irrigation water on three groups viz. one group from putrescine - treated plants, one group from GA_3 - treated plants and other group from control plants. Salinity level of the soil was maintained by checking the electrical conductivity of the soil extract every week. Salinity level was maintained by adding required amount of NaCl to the irrigation water. Sufficient number of additional pots containing plants were maintained to check the salinity level as well as for collecting samples for various analyses during the experiment. From each treatment, 20 replicates were earmarked for recording the data on the yield parameters. All pots received about one litre water every alternate day. Plants were also given half strength Hoaglands' solution (Epstein, 1972), 500 ml, once in 15 days. Putrescine or GA_3 spray was repeated a month after the first spray. The maximum-minimum temperature during the growth period were 32 $\pm$ 3°C and 21 $\pm$ 2°C respectively.

Growth measurements

Plants were harvested at desired intervals, washed with water, blotted and shoots and roots were seperated. Immediately after seperating, length and fresh weights of shoot and root systems were recorded. The seperated shoots and roots were dried at 80°C for 72 h and dry weights were determined. The growth measurements, the first two and the subsequent ones

were made at an interval of 5 and 10 days respectively from the day of imposition of salt treatment.

Leaf area measurements

Leaf area measurements were made planimetrically. Total leaf area per plant is tabulated and expressed as cm^2.

Plant samples were collected at desired intervals, washed with distilled water, blotted and were seperated into shoot and root systems. Known weights of shoot and root tissues were then stored in deep freeze for various analyses. For dry material, plant tissues were oven-dried and powdered using a mortar and pestle.

Extraction of Na^+, K^+ and Cl^- ions

The procedure employed for the extraction of Na^+, K^+ and Cl^- ions was the one described by Wignarajah *et al.* (1975b) with some modifications. Known weight of (200 mg) dry powdered plant material was extracted thrice with boiling deionized water and the supernatant was collected after centrifugation at 6,000 g for 10 min. The residue obtained was then extracted with 30% (v/v) nitric acid at 90°C for one hour. The suspension was cooled and the supernatant was collected by centrifugation at 6,000 g for 10 min. The extraction of the residue with nitric acid was repeated twice. Supernatants were pooled and made upto a known volume.

Estimation of Na^+, K^+ and Cl^- ions

Na^+ and K^+ ions were estimated flamephotometrically. Chloride was estimated following the procedure of Clark (1950). 10 ml of the extract was taken in a beaker and to that 5 drops of 1% diphenyl carbazone indicator was added. The extract was then titrated against 0.0141N mercuric nitrate till a persistent blue-violet colour developed. The quantity of mercuric nitrite required for colour development was recorded and chloride content of the solution was calculated by the following equation

$$Cl^- \text{ in ppm} = \frac{V \times 35,460\ N}{S}$$

Where V = ml of mercuric nitrate consumed,

N = normality of mercuric nitrate and

S = ml of sample (extract).

Total Chlorophyll estimation

Fifty milligrams of fresh leaf tissue was homogenized in 80% acetone and centrifuged at 3,000 g for 10 min. Chlorophyll levels in the supernatant, after appropriate dilutions, were determined spectrophotometrically according to the method described by Harborne (1984).

Extraction and estimation of total protein

Two hundred mg powdered material was suspended in 10 ml 0.3 N KOH and incubated for 18 h at 37°C. The supernatant was collected by centrifugation of the suspension at 10,000 g for 15 min and the residue was washed four times with KOH. The supernatants were pooled and the final volume was made upto 50 ml with distilled water and an aliquot of this extract was used for the estimation of protein content following a modified Lowry's method (Hartree, 1972) using bovine serum albumin as standard.

Extraction and estimation of proline

The method described by Bates et al. (1973) was employed to extract and estimate proline content of the tissue. A known weight (250 mg fr.wt.) of shoot or root tissue was homogenized in 10 ml of 3% aqueous sulfosalicylic acid and the homogenate was filtered through Whatman No. 2 filter paper. Two ml of the filtrate was reacted with two ml acid ninhydrin (625 mg ninhydrin in 15 ml glacial acetic acid and 10 ml 6 M phosphoric acid) and two ml glacial acetic acid in a test tube at 100°C for one hour and the reaction was terminated by keeping the tubes in ice bath. The reaction mixture was extracted with six ml toluene. The chromophore containing toluene was aspirated from the aqueous phase, warmed to room temperature and the absorbance read at 520 nm

using toluene for blank. The proline concentration of the extract was determined from a standard curve of proline prepared according to the above procedure.

Extraction and estimation of total quaternary ammonium compounds

The modified periodic method described by Grieve and Grattan (1983) was adapted for the extraction and quantification of total quaternary ammonium compounds. Dry powdered plant material (500 mg) was mechanically shaken with 20 ml of deionized water for 24 h at 25°C. The sample was then filtered and a known aliquot of filtrate was diluted with equal volume of 2 N H_2SO_4. Aliquots of 0.5 ml were measured into glass centrifuging tubes and cooled in ice for one hour. Cold KI-I_2 reagent (0.2 ml), prepared by dissolving 1.57 g of iodine and 2.0 g KI in 10 ml water was added and the reactants were gently stirred. The tubes were stored at 4°C for 16 h and then centrifuged at 5,000 g for 15 min at 2°C. The supernatant was carefully aspirated and periodic crystals were dissolved in 9.0 ml of 1,2-dichloroethane. After two hours the absorbance was measured at 365 nm and the total quaternary ammonium compounds were determined. Glycinebetaine prepared in 1 N H_2SO_4 was used as reference standard.

Extraction and seperation of indole acetic acid

Indole acetic acid was extracted following the method of Mc Dougall and Hillman (1978) with some modifications.

A known weight (about 10 gm fr.wt.) of plant material was homogenized in 80% chilled methanol (1:5 w/v) for 5 min and kept overnight at 6°C in darkness. An antioxidant 2,6-di-tert-butyl-4-methylphenol (100 mg/lit) was included in the extraction medium. The methanolic extract was centrifuged at 6,000 g for 15 min and the residue was extracted twice using the same solvent, each time for a period of six hours. All supernatants were mixed and the organic phase is removed by rotary film evaporator at 35°C to yield concentrated aqueous phase. The pH of the aqueous layer was adjusted to 7.5 with 1 N sodium bicarbonate and partitioned twice with peroxide free diisopropyl ether (1:3 v/v). The aqueous phase was then acidified to pH 3.0 with 2 N HCl. The acidified extract was partitioned three times against equal volumes of diethyl ether. The basic and acidic fractions were pooled together, dried over anhydrous sodium sulphate and evaporated in vacuum. The residue was taken up in 2 ml methanol.

Thin layer chromatography

The above methanolic extract was streaked at the origin of 20 x 20 cm silica gel G plate (250 μ thickness). Guide spots of standard IAA and of extract were made at both the sides of streak. After chromatographic development in benzene : acetone : pyridine (60:39:1) solvent system, the area of sample streak was covered and the guide spots were located after spraying with modified Salkowski reagent (Gordon and Weber, 1951). The appropriate area corresponding to

standard IAA was scraped from the plate and eluted with 3 x 5 ml of 50% aqueous ethanol. The ethanolic extracts were evaporated to dryness and redissolved in 0.5 ml of 50% aqueous ethanol.

Estimation of IAA

The TLC seperated IAA fraction was evaporated to dryness in a small vial and silylated with BSA (bis-trimethylsilyl-acetamide) (Bandhurski and Schulze, 1974). After silylation 2 µl samples of the extract of IAA derivated with BSA were injected into a Hewlett Packard model HP 5840A gas chromatograph equipped with flame ionization detector and with a 50 x 0.3 cm stainless steel column packed with 10% UCW - 98 on chromosob W-AW 80/100 mesh. The column temperature was programmed from 150°C to 250°C at the rate of 15°C min^{-1}. The injection and detector temperatures were at 250 and 300°C respectively and the flow rate of nitrogen carrier gas was 25 ml min^{-1}. The peak area on the recorder chart was mwasured and the amount of IAA in the extract was determined from IAA standard curve.

IAA loss during the extraction and purification steps was determined by passing a known amount of standard IAA sample through the above procedure. Recovery of IAA was 59 ± 4.1% (mean ± standard error). IAA values presented here have been corrected for extraction and purification losses.

Extraction and seperation of gibberellin - like substances

Extraction and purification of gibberellin - like substances were carried out according to the procedure of Jones and Lang (1968). A known weight (about 10 g fr.wt.) of plant material was frozen and homogenized with 80% methanol (100 ml) for 10 min. The homogenate was incubated at 6°C for 12 h and was filtered. The residue was re-extracted with 100 ml of 80% methanol for 6 h at room temperature and filtered. Lipid material present in the extract was removed by mixing petroleum ether (boiling range 30-60°C) with the methanolic extract. The organic solvent in the extract was evaporated on a flash evaporator at 50°C. The remaining aqueous phase was adjusted to pH 9.5 with 1 N NaOH and partioned twice against ethyl acetate. This ethyl acetate fraction was discarded and the remaining aqueous phase was acidified to pH 2.5 with 1 N HCl and partioned four times against equal volumes of ethyl acetate. The combined acidic ethyl acetate fraction was dried over anhydrous sodium sulphate before further purification by thin layer chromatography.

Thin layer chromatography

Extracts were purified by TLC prior to bioassay. Acidic fractions were reduced to dryness, redissolved in a small volume (2 ml) of ethyl acetate and streaked to the origin of 20 x 20 cm silica gel plates as mentioned earlier. The plates were developed in acetone : benzene : formic acid (48 : 50 : 2).

Following development, the plates were divided into 10 equal zones between origin and solvent front. Each zone was scraped off and eluted three times with 5 ml of ethyl acetate. The eluates were reduced to dryness and redissolved in 5 ml ethanol.

Bioassay

Samples of TLC eluates, after evaporating ethanol, made upto a known volume with sterile distilled water and assayed with barley endosperm bioassay (Nicholls and Paleg, 1963) after suitable modification. Barley seeds (selected seeds of uniform size) were soaked in a freshly prepared sterilizing solution of 5% sodium hypochlorite in a stoppered flask for 3 h at 25°C, and washed ten times with 100 ml lots of sterile distilled water. The disinfected grains were incubated in sterile distilled water for 24 h at 30°C. Seeds were then cut transversely 3 mm from the distal end with a sterile scalpel and the embryo-containing fragment discarded. The remaining endosperm portions were placed in groups of 4 each in small sterile specimen vials containing 1.0 ml test solution (plant extract or distilled water, pH 5.8). Each vial contained 500 µg streptomycin to prevent the contamination from microorganism. The stoppered vials were incubated at 30°C for 48 h. All experimental manipulations were carried out under aseptic conditions. Samples of ambient media were then assayed for reducing sugars by the procedure of Somogyi (1952).

The amount of gibberellin - like substances of the extract was calculated from standard curve prepared with authentic gibberellic acid (GA_3) following the above bioassay procedure. Percentage recovery of known amounts of authentic gibberellic acid standard after the above detailed extraction and purification steps was 67 ± 3.5% (mean ± SE). Amount of GA - like substances presented here has been corrected for extraction and purification losses.

Extraction and seperation of abscisic acid

The method employed for the extraction and purification of abscisic acid (ABA) is that of Downton and Loveys (1978) with certain modifications. Frozen shoot or root tissue (about 10 gm fr.wt.) was homogenized for 10 min in 150 ml of chilled extraction medium (methanol, ethylacetate, acetic acid; 50 : 50 : 1, v/v) containing 100 mg/l 2,6-di-tert-butyl-4-methyl phenol as an antioxidant. An internal standard of 2-trans-ABA was added at the rate of 10 µg/gm fresh weight to the homogenate (Lenton et al. 1971) and was stored in darkness for 24 h at 6°C and filtered. The residue was re-extracted twice, each time for a period of 6 h with 75 ml of the same extracting solvent. The pooled filtrates, after the addition of 35 ml of deionized water, was evaporated to aqueous phase under vacuum at 40°C. The aqueous phase was adjusted to pH 8.5 with 0.1 M NaOH and partitioned

four times against 40 ml redistilled diethyl ether. The pH was then adjusted to 2.5 with 0.1 M HCl and the aqueous extract was partitioned four times against 40 ml ethyl acetate to extract the free form of ABA.

Thin layer chromatography

Extracts of free ABA were applied on glass plates coated with silica gel G (300 μ thickness) and developed in toluene : ethyl acetate : acetic acid (25 : 15 : 2). Marker spots of authentic mixed isomer ABA (Sigma, USA) were visualized under UV light with out exposing the samples. The zone corresponding to authentic sample was scraped off from the chromatograms and eluted with 9 : 1 (v/v) acetone : methanol mixture.

Estimation of ABA

A known volume of extract, after TLC purification, was derivatized with BSA (bis-trimethyl silyl acetamide) as described by Davis *et al.* (1968). After derivatizing with BSA 2 μsamples of extract or authentic ABA were injected into a Hewlett Packard model HP 5840 A gas chromatograph equipped with flame ionization detector and with a 50 x 0.3 cm stainless steel column packed with 10% UCW - 98 on chromosob W-AW 80/100 mesh. The column temperature was programmed from 150°C to 250°C at the rate of 15°C min^{-1}. The injection and detector temperatures were at 250 and 300°C respectively, and the flow rate of nitrogen carrier gas was 25 ml min^{-1}. The peak area on the recorder chart was

measured and the amount of ABA in the extract was determined using an ABA calibration curve. Recovery of standard ABA was 61 ± 2.1% (mean ± SE). ABA values presented here have been corrected for extraction and purification losses.

Preparation of enzyme extract

Fresh plant material was homogenized in cold 0.2 M borate buffer (pH 8.6; 8 ml/g fr.wt.), along with a pinch of glass powder, for 5 min using a chilled mortar and pestle. The homogenate was filtered through four layers of cheese cloth and centrifuged at 10,000 g for 10 min at 0°C. The protein present in the extract was precipitated by mixing it with chilled acetone at a ratio of 1:2 v/v at 0°C. The precipitated protein was sedimented by centrifugation (15,000 g) at 2°C for 15 min and was dissolved in different buffers for various enzyme assays (0.02 M phosphate buffer, pH 6.1, for IAA oxidase; 0.05 M acetate buffer, pH 4.6, for acid invertase; 0.05 M acetate buffer, pH 4.5, for amylase; 0.05 M acetate buffer, pH 5.0 for cellulase; 0.1 M Tris-HCl buffer, pH 8.0, for pectin lyase and proline oxidase).

IAAoxidase assay

IAA oxidase activity was determined by the method of Gordon and Weber (1951). The reaction mixture (4.0 ml) consisting of 1.0 ml enzyme extract, 198 µg $MnCl_2$, 162 µg

2,4-dichlorophenol, 200 µg IAA and 1.0 ml 0.2 M phosphate buffer, pH 6.1, was incubated for 30 min at 30 ± 1°C in dark. After incubation 2.0 ml of the reaction mixture was added to 4.0 ml modified Salkowski reagent and the colour was allowed to develop for 25 min. The absorbance of the solution was measured at 530 nm. Boiled enzyme was used in preparing blank. The enzyme unit is defined as the amount of enzyme required to oxidase 1 µ mole of IAA per hour under the assay condition and the enzyme activity is expressed as units per mg protein.

Total amylase assay

Total amylase activity was determined by the method of Bernfeld (1955). The assay system (2.0 ml) consisted of 0.5 ml 0.1 M acetate buffer (pH 4.5), 0.5 ml 1% soluble starch and 1.0 ml enzyme. The reaction was carried out at 30 ± 1°C for 60 min and an aliquot of 1.0 ml was estimated for the amount of maltose released using 3,5-dinitrosalicylic acid. The enzyme unit is defined as the amount of enzyme required to liberate 1 µ mole of maltose per hour under the assay condition and the activity is expressed as units per mg protein.

Acid invertase assay

The activity of acid invertase was measured by estimating the reducing sugars produced in the assay system after an

incubation period of one hour. The assay system (2.0 ml) contained 1.0 ml of 0.1 M acetate buffer (pH 4.6), 0.5 ml 2 % sucrose and 0.5 ml enzyme extract. The reaction mixture was incubated at 30 ± 1°C for 60 min and the reaction was terminated by keeping it in a boiling water bath for 10 min. An aliquot from the reaction mixture was used for the estimation of reducing sugars according to the procedure of Somogyi (1952). The enzyme unit is defined as the amount of enzyme required to produce 1 µmole glucose per hour under the assay condition and the enzyme activity is expressed as units per mg protein.

Cellulase assay

Cellulase was assayed as per the method followed by Trivedi and Rao (1979) 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) in 0.05 M acetate buffer (pH 5.0). The reaction mixture was incubated at 37°C for 30 min. The reaction was stopped by heating the tubes in a boiling water bath for 5 min and an aliquot of the reaction mixture was used to determine the reducing sugars released according to the method of Somogyi (1952). One unit of enzyme is defined as the amount of enzyme required to release 1 µmole of reducing sugars per hour under the assay condition and the activity is expressed as units per mg protein.

Pectin lyase assay

Pectin lyase activity was measured according to the method of Wijesundera et al.(1984). The reaction mixture contained 3.0 ml of 0.25 % (w/v) citrus pectin in 0.1 M Tris-HCl buffer (pH 8.0) and 0.5 ml of enzyme extract. The reaction was carried out at 35 ± 1°C for 45 min. After 45 min of incubation, 1.0 ml samples are added to 6.0 ml TBA (thio barbutiric acid) reagent (5.0 ml of 0.01 M TBA and 1.0 ml 1 M HCl) and kept in a boiling water bath for 60 min. The tubes were then cooled to room temperature and the absorption was read at 550 nm. One unit of enzyme is that amount which causes an increase in one optical density at 550 nm per hour under assay condition and enzyme activity is expressed as units per mg protein.

Proline oxidase assay

Proline oxidase was assayed following the method of Huang and Cavalieri (1979). The 5 ml reaction mixture contained 50 m M Tris-HCl (pH 8.0), 2.5 mM $MgCl_2$, 0.5 mM FAD, 1mM Phenazine methosulfate, 0.06 mM dichlorophenol indophenol, 5 mM proline and enzyme extract (1.0 ml). The reaction was monitored at 600 nm at 30 ± 1°C using proline to initiate the reaction. One unit of enzyme is defined as the amount of enzyme which causes a change in optical density of 0.01 per min at 600 nm under the assay condition and enzyme activity is expressed as units per mg protein.

Agmatine deiminase assay

Agmatime deiminease was assayed according to the method followed by Desai and Mehta (1985). The assay system of 4 ml contained 25 mM phosphate buffer (pH 6.5), 2.5 mM agmatime sulphate and 1.0 ml of enzyme. The reaction mixture was incubated at 37°C for 1 h and the reaction was terminated by adding 0.5 ml of 10% TCA. The precipitate formed was removed by centrifuging at 10,000 g for 10 min and an aliquot of the supernatant was used for the estimation of the N-carbamyl putrescine formed using the method of Archibald (1944), The enzyme unit is defined as the amount of enzyme required to liberate one μmole of N-carbamyl putrescine per hour under assay conditions and the enzyme activity is expressed as units per mg protein.

Protein content of enzyme extract was determined following the procedure of Hartree(1972) using bovine serum albumin as standard.

Extraction and estimation of polyamines

Polyamines were extracted and estimated following the method of Inoue and Mizutani (1973) after some modifications. Polyamines were extracted by homogenizing 1.0 g fresh tissue in 5 ml of cold 5 % perchloric acid using a chilled mortar and pestle. The homogenate was then centrifuged at 10,000 g for 15 min and the pellet was washed with 3 ml of 5% perchloric acid. The supernatant and washings were combined

and applied to 1x6 cm column of Dowex-50 W (200-400 mesh, 8 % cross-linked). The column was washed with 40 ml of 0.1 M sodium phosphate buffer, pH 8.0, containing 0.7 M NaCl and then with 10 ml of 1 N HCl. Polyamines were later eluted from the column with 15 ml of 6 N HCl. The acid eluate was collected in a beaker containing 0.1 mg EDTA before evaporating it to dryness at 60°C. The residue thus obtained was dissolved in 0.5 ml of 0.05 N HCl. Aliquots of 10 to 20 µl were spotted on 250 thick air dried TLC plates precoated with sigmacell (non-crystalline cellulose). Polyamine standards treated similarly were co-chromatographed with the samples and the plates were developed in methyl celloslove : propionic acid : water (70:15:15) saturated with NaCl. After removal of the solvent, plates were sprayed with ninhydrin reagent prepared according to Inoue and Mizutani (1973) and heated at 70°C for 60 min to develop the colour. Ninhydrin reagent contained 500 mg ninhydrin, 50 mg cadmium acetate, 5 ml water, 2.5 ml acetic acid and 42.5 ml acetone. The sports corresponding to polyamines were scrapped and extracted for 30 min with 5 ml of solvent containing 0.2 g of cadmium acetate, 10 ml of water, 40 ml of ethanol and 50 ml of acetic acid. The optical density of the extract was measured at 505 nm and was compared with that of standards.

Grain yield

Yield parameters analysed included (a) total number of filled grains; (b) total number of unfilled grains; c) total

weight of filled grains per plant and d) weight of 1000 grains.

The values shown for extension growth, fresh weight and dry weight are the means of 15 measurements. In the case of leaf extension growth and total leaf area each value is the mean of 10 measurements. The data for yield parameters are the means of 20 replicates. All the growth and yield data except that of leaf extension growth have been subjected to student's t-test (Snedecor,1959). The values reported for various chemical and biochemical analyses are the average of 2 to 5 replicates $\pm$ SE.

The growth period mentioned in the results are days from the time of imposition of salt treatment unless otherwise stated.