MATERIAL AND METHODS

Material and Methods.

The seedings of 2 varieties of chilli (Capsicum annuum) were raised in the field for 30 days in fertile soil. After complete growth of the seedlings for 45 days, with normel watering, the saline treatment was commenced.

The plants were stressed with increasing concentrations of NaCl. Salinity Viz control, 0.05 M 0.1 M, 0.15M and 0.2M. The plants were treated thrice a week with alternate watering, the plants. To avoid accumulation of excess salts and to check the loss of water due to evaporation in the medium the plants were watered regularly alternating with salt treatment.

The control plants recieved only water. After completion of 60 days treatment the plants were carefully uprooted. washed under tap water throughly and then washed with distilled water. All the Parts of the Plant leaves, stem and roots. were separated and blotted to dry. Then the fresh material was used for chlorophyll, TAN, Nitrite, and Nitrate Reductase estimation. And the remaining material was kept in the Oven for drying for the analysis.

1. Photosynthetic Pigments.

The chlorophylls from the leaves were estimated following the methods of Arnon (1949) and Kirk and Allen (1965) respectively. For this young and mature leaves were washed first with tap water and then with distilled water, blotted to dry and cut into small pieces. This material was homogenised in 80% acetone and filtered through whatman No.1 filter paper under suction. The residue was washed throughly 2-3 times with 80% acetone, collecting all the washings in the same containes. Final volume was made to 100 ml with 80% acetone. Absorbance was read at 663 and 645 for chlorophyll a and chlorophyll b respectively on spectrophotometer. The chlorophyll a chorophyll b and total

chlorophylls were calculated by the formula suggested by Arnon (1949).

Chlorophyll "a" 12.7 x A 663 - 2.69 x A 645 = x

Chlorophyll "b" 22.9 x A 645 - 4.68 x A 663 = Y

Total chlorophyll 8.02 x A 663 + 20.2 x A 645 = Z

2. Titralable acid number

The method described by Thomas and Beevers (1949) was followed to determine TAN. Fresh leaf tissue was washed andrinsed with distilled water and blotted to dryness. It was accurately weighed (8.5 g) and boiled for half an hour in 100 ml distilled water. After cooling, it was filtered through cheese cloth and volume was made to 50 ml with distilled water. The filterate was then titrated against standardized NaOH (N/40) using phenolpthalein as an indicator. TAN represents the number of ml of decinormal NaOH required to neutralise the acid present in 100 g fresh tissue. TAN value was calculated using the following formula.

$$\frac{\text{Vol. of oxalic acid taken for titration}}{\text{Titration reading ml}} \times \frac{\text{Total vol. of Extract ml}}{\text{Weight of Plant material in gm}} \times \frac{\text{Extract tit.}^{n} \text{ reading ml}}{\text{Vol. of Ext. taken for tit.}^{n}} \times \frac{100}{4}$$

3. Carbohydrates

The sugars were estimated following the method of Nelson (1944). The soluble carbohydrates were extracted from 0.5 g oven dried seedling tissue (plant material) with 80% alcohol. The extract was filtered through Buchners funnel using Whatman no 1 filter paper. The filterate thus obtained was condensed on water bath to about 5 ml. To this 2.3g lead acetate and potassium

oxalate (1:1) were added, 50 ml of distilled water was added and aliquot was filtered. The volume of filterate was measured and it served as an extract for determination of reducing sugars. A known volume of this extract (20 ml) was hydrolysed with 2 ml conc HCl by autoclaving at 15 lbs atm pressure for half an hour. The content was cooled neutralized with anhydrous sodium carbonate and filtered. The volume of the filterate was measured and this filterate was used for the estimation of total (reducing + non reducing) sugar.

4. Starch

For estimation of starch, the insoluble residue obtained at the beginning after filtering the alcoholic extract was transfered to a 100 ml (capacity) conical flask with 50 ml distilled water and 5 ml conc. HCl. The same was hydrolysed at 15 lbs atmosphere pressure for half an hour and cooled to room temperature neutralised by addition of anhydrous Na_2CO_3 and filtered. The volume of the filterate was measured as this contains reducing sugars (glucose) formed as a result of hydrolysis of starch. These sugars represent the starch content in this residue.

The sugars from all these filterates were estimated by determining the reducing power by employing arsenomolybdate reagent introduced by Nelson (1944).

For the Colorimetric determination of the cuprous oxide formed in the oxidation of sugars by alkaline Copper tartarate reagent. For this 0.5 ml aliquots and 0.1 ml aliquot for starch were taken in test tubes along with different concentration of standard glucose soln (0.1 mg/ml) in other test-tubes. To this, requisite amount of distilled H_2O was added to make

final volume 10 ml. In case of blank instead of filterate or standard glucose, distilled water was added to begin with the reaction. One ml of somogys alkaline copper tartarate reagent (4g $CuSO_4$ $5H_2O$, 24 g anhydrous Na_2CO_3, 16 g Na+K tartarate and 180 g anhydrous Na_2SO_4 dissolved in 1 litre distilled water) was added in each test tube and all these reaction mixtures were transfered to boiling water bath for 10 min. After cooling to room temperature 1 ml Arsenomolybdate reagent (125 g NH_4MoO_4 in 450 ml water to which 21 ml conc H_2SO_4 were added followed by 3 g Na_2HASO_4, $7H_2O$ dissolved in 25 ml water. These ingradients were mixed well and digested for 48 hrs. at 37° C in incubator before use] was added to each reaction mixture which were further diluted to 10 ml with distilled water. After 10 min. absorbance was read at 560 nm. on spectrophotometer.

Using calibration curve of standard glucose the sugar percentage in the above three fractions were calculated values of soluble sugars are expressed as g $100g^{-1}$ dry tissue.

5. Total Polyphenols

Total Polyphenols were determined according to the method of Follin and Dennis (1915). Dry leaf material (0.5 gm) was homogenised in 30 ml of 80% acetone and filtered through Buchners funnel. The residue was washed several times with 80% acetone and the final volume was made 100 ml with 80% acetone. Two ml extract along with a series of standards (std. tannic acid, 0.1 mg per ml.) were taken in seperate. Nesseler's tubes and to each tube 10 ml of 20%. Na_2CO_3 and 2 ml Follin Dennis reagent (100 g Na tungstate mixed with 20 g Phosphomolybdic acid in about 800 ml distilled water to this 200 ml 25% phosphoric acid was added and

the mixture was refluxed for 2.1/2 hrs. to room temperature and volume made to 1000ml with distilled water) was added. Final volume was made 50 ml with distilled water. After 20 min. absorbance was read at 660 nm using reagent blank. Total Polyphenols were calculated with the help of standard curve of Tannic acid and expressed as g 100 g^{-1} dry tissue.

6. Proline

Proline content from various plant parts of chilli species was determined according to the method of Bates et al., (1973). For this, 0.5 g oven dried Plant material was homogenised in 10 ml Sulphosalicylic acid (3%) throughly and then filterate was reacted with 2 ml glacial acetic acid and 2 ml acid ninhydrin reagent prepared by warming 1.2 g ninhydrin in 30 ml glacial acetic acid and 20 ml 6M Phosphoric acid with agitation, cooled and stored at 4°C) in a test tube for 1 hour at 100°C in boiling water bath similar procedure was also followed for another set of test tubes containing various concentrations of standard proline solution (0.1 mg proline/ml). After boiling the reaction was terminated by transfering the test tubes immediately to ice bath. To this 4 ml of Tolune was added and mixed vigourously for 15-20 sec. Reaction mixtures were then brought to room temperature and absorbance of toluene chromophore was measured at 520 nm using toluene, blank. Proline concentration was calculated from calibration and final volume were expressed as g 100 g^{-1} dry tissue.

7. Total Nitrogen

Plant grown under different salt treatment were estimated for nitrogen following the method of Hawk et al.,(1948) Ovendried

Powdered plant material (0.1 g) was taken in Kjeldalhs flask with a pinch of micro salt (200 g K_2SO_4 + 5 g $CuSO_4$ dehydrated) and to it 5 ml H_2SO_4 (1:1) was added. Few glass beads were added to avoid bumping and the material was digested on low flame. After complete digestion a faint yellow solution was obtained which was cooled to room temperature and transfered to Volumetric flask, diluted to 100 ml with distilled water.

In Nesseler's tubes 1 ml of plant extract and different conc of standard ammonium sulphate soln (0.2336 of Ammonium sulphate dissolved in distilled water and few drops pf H_2SO_4 were added. The volume was made 1000 ml with distilled water. This solution contains 0.05 mg of nitrogen per ml.) are taken. In control tube 1 ml distilled water was taken. To this 1 drop of 8% $KHSO_4$ was added and volume was made 35 ml with distilled water. To this 15 ml Nessclers reagent was added. (Reagent A: 7 g KI + 10 g Hg I_2 in 40 ml d/w) (Reagent B: 10 g NaOH in 50 ml water. A and B are to be mixed in proportion of 4:5 at the time of estimation. The reaction between the sample and the reagent gives the product $NH_4Hg_2I_3$ which has has Orange brown colour. This colour was measured after 15 min at 520nm on spectrophotometer.

Crude proteins were calculated by multiplying the total nitrogen value by factor 6.25.

8. I Nitrate reductase (EC 1.6.6.1)

Activity of this enzyme in Vivo was determined following the method described by Jaworski (1971). The leaf tissue was incubated in the medium containing 1 ml, 1 M KNO_3, 2 ml, 5% n-Propanol, 5 ml 0.2 M Phosphate buffer PH 7.5 and 2 ml 0.5% titron-X-100 for 1 hour in dark under anaerobic conditions after

1 hour, 1 ml of reaction mixture was taken out and mixed with 1ml, 1% sulfanilamide in 1 ml HCl and 1 ml 0.02% NEEDA (N-1

Naphthylethylene diamine dihydrochloride). The absorbance was read at 540 nm on spectrophotometer (Elico).

Standard Curve was prepared with 0.03 mM KNO_2 (0.0026 mg KNO_2/ml distilled water) while mixture of 1 ml incubation medium, 1 ml Sulfanilamide and 1 ml NEEDA served as a blank.

Enzyme activity is expressed as nm NO_2 liberated g^{-1} fresh tissue hour.

9. Nitrite reductase (Ec 1.6.64)

The activity of enzyme nitrite reductase (NR) was determined following the same method described already for NR except that KNO_3 was replaced by 0.3 mM KNO_2 in the incubation method and the incubation was done in the light. Changes in KNO_2 present in the incubation medium were determined by reading the Optical density of the reaction mixture containing 1 ml incubation medium, 1 ml sulfanilamide and 1 ml NEEDA. The difference between the two readings one at 0 min and the other after the enzymatic reaction gives an amount of KNO_2 utilized by the enzyme (reduced).

10. MINERAL NUTRITION:

Procedure for Acid digestion method

Take 0.5 gm ovendried plant material i.e. stem leaves, Roots. Then it was transfered to 100 ml capacity beakers. 20 ml of Conc HNO_3 was then poured into the beaker. Then allow it to initiate for 30 min. Then heat the beakers gradually to dissolve

each and every particle of material. Cool to room temperature. Then add 10 ml 72% Perchloric acid.

Again heat gradually to a colourless solution condense to the volume upto 1 to 2 ml cool it. then add distilled water and adjust the volume to 100 ml. Keep this extract overnight on Next day filter the extract through Whatman filter paper No.1 and use as source of plant extract for Iron and Phosphorus.

Calculation : 1 ml std. Fe solution = 0.1 mg Fe.

1 ml std. P solution = 0.025 mg P.

1. Sodium, Potassium

These cations were estimated according to standard flame photometric process employing Toshniwal flame photometer for standardization of various concentrations of Na (20ppm), K (20ppm) and Ca (200 ppm) from NaCl and KCl and CaCl respectively were prepared using these standard solutions, standard curves for these elements were prepared using flame photometer with specific filters. The plant extract was subjected to same procedure. Calcium, Magnesium, Copper, Zinc and Manganese are estimated by Atomic Absorption Spectrophotometer.

2. Phosphorus

For estimation of Phosphorus method of Sekine et al., (1965) was followed. Here Phosphorus reacts with "Molybdate Vanadate Reagent" to give yellow colour Complex. By estimating colorimetrically the intensity of the colour developed and by comparing it with the colour intensity of known ~~and by comparing it with the colour intensity to known~~ standards, Phosphorus content was estimated.

To 2 ml of acid digest in a test tube 2 ml of 2N HNO_3 were added and adjust Vol. 10 ml with distilled H_2O followed by 1 ml of Molybdate Vanadate reagent (A-25 g Ammonium molybdate in 500 ml of distilled water, B-1.25 g Ammonium Vanadate in 500 ml in 1N HNO_3 A and B are mixed.) The ingredients were mixed well and allowed to react for 20 minutes. After 20 minute colour intensity was measured at 420 nm using a reaction blank containing no phosphorus. Calibration Curve of standard Phosphorus was prepared from standard Phosphorus solution (0.110g KH_2PO_4 per litre = 0.025 mg P^{5+} ml^{-1}) taking different concentrations 0.025, 0.05, 0.1, 0.2 and 0.4 mg of Phosphorus). Others steps being essentially similar to the one described above with the help of standard curve, amount of Phosphorus in the Plant material was calculated.

3. Chlorides

Chlorides were extracted according to the method described by Inamul Huq and Larher (1983) with slight modifications and estimated according to the method of Chapman and Pratt (1961). The chlorides were extracted in distilled water at 45°C for 1 hour and add hot distilled water was added to prevent drying. After cooling the extract was filtered through a layer of cheese cloth. The filterate was collected in 50 ml. volumetric flask and volume was made with distilled water. For this 10 ml extract was taken for titration against standardized $AgNO_3$. Few drops of acetic acid (20%) Soln (dilute 200 ml conc. acetic acid with 800 ml of distilled water) were added to the filterate until the pH of the solution was 6 to 7. Then five drops of Potassium chromate soln

added

(1%) was added and titrated with standardized 0.05 N silver nitrate. (Dissolve 8-5) g AR grade $AgNO_3$ in distilled H_2O Transfer to 1 liter volumetric flask and make up to volume with distilled water) until the first permenant reddish brown colour appears.

Standardization : Put 10 ml of 0.1N Sodium Chloride standard into Erlemeyer flask and add 50 ml of distilled water. Titrate with the Prepared Silver Nitrate Solution.

1 ml 0.05N $AgNO_3$ = 1.77 mg of chlorides

4. To Estimate Iron in Plant tissue

Aim : To estimate Iron from plant tissue

Principle : Iron is one of the micronutrient in plants. It plays an important role in oxidation Reduction type of Reactions. It is also involved in cytochrome and cytochrome oxidase. Enzyme is the respiratory electron transfer.Iron is also essential for chlorophyll synthesis.

Iron in the plants exsist usually in Ferric (Fe^{+++}) form. It can be estimated from ovendried plant material following method by Durie*etal*(1965) which is in Ferric form in the acid digest of plant material is reduced to ferrous (Fe^{++}) ions. Ferrous ions developed colour with O phenon throline in alkaline medium. The intensity of red colourisation is directly proportional to the Iron content in the plant extract. The optical density of redcoloured complex is read at 520 nm on spectrophotometer. By comparing this absorbance with that of the standard Iron solution. Iron in plant material can be calculated.

EFFECT OF VARIOUS CONC. OF NaCl on PC1XDB Hybrid variety.

fig no 2.

EFFECT OF VARIOUS CONC. OF NACL ON ~~PCMXDB~~ LXDB Hybrid variety.

Fig no③