CHAPTER 3

MATERIAL AND METHODS
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CULTURE:

For studied presented in chapter III experimental plants were raised in soil culture under pot culture conditions.

CONTAINER:

Plants were raised in 8" clay flower pots with a central drainage hole. The inner surface of the pots along with top 3" of outer surface, was lined with acid washed and distilled water rinsed polythene provided with a central hole superimposed on the drainage hole of the pot. The soil was retained in the pots by means of a pad of glass wool kept beneath the rim of an inverted watch glass placed above the drainage hole. The watch glasses and glass wool were purified by boiling in 1:1 hydrochloric acid and rinsing with distilled water. About 2.5 kg soil were used in each pot.
WATER:

The distilled water required for culture work and analytical work except for iron and manganese were obtained from water stills manufactured by Gansons Private Ltd., Mumbai. For the determinations of iron and manganese, corning glass distilled water were used.

RAISING OF PLANTS:

All the plants were raised from distilled water washed seeds or grains shown directly in the sand. 2 cm deep holes were made with an acid washed clean glass rod of 3 mm diameter and seeds were put in these holes, covered loosely with sand of the same pot. As many as 25 seeds were shown in each pot. After the seed emergence plants were thinned to a uniform number in each pot subsequent thinning was done whenever needed.

LAYOUT:

For each treatment there were six pots. The pots were arranged in 3 blocks A, B and C. In each block the treatment were completely randomized. The experimental pots were arranged in north-south direction and were kept raised from the ground at a height of one and half feet avoiding any surface contact of the drainage holes with the ground of eliminate any contamination.

SOIL:

Soil samples were collected from near by localities of Kanpur district, in clean polythene bags after surface scrapping and brought to the laboratory.
APPLICATION OF VERMICOMPOST AND AZOTOBACTER:

Soil was separately mixed with required amount of vermicompost and Azotobacter as first dose as described in chapter IV. Thereafter it was air-dried thoroughly ground and mixed. For thoroughly mixing, required amount of vermicompost and Azotobacter were mixed with small amounts of soil, divided and mix again and again. Then these amended soils were mixed with bigger amounts of soil similarly and finally these soils were mixed with bigger lots of calculated soils required for experiments. Soil mixing was done on separate clean alkathene sheets to avoid any contaminations. Mixed soils were filled in pots as described earlier. About 2 ½ kg soil were filled in each pots. The details of procedure of soil preparation and dose of vermicompost and Azotobacter application is being described in chapter IV.

SAMPLING TECHNIQUE:

Sampling was generally started at 8 A.M. and completed in an hour. All samples were drawn at the same time put in washed polythene bags and placed in refrigerator at about 5°C until needed as early as possible. The 3 blocks a, b and c were sampled at the same time.

ANALYTICAL:

PLANT ANALYSIS:

For analysis, washed finally chopped and mixed plant material was used. For the determination of ascorbic acid and chlorophyll contents and for assay of catalase and peroxidase, fresh
material was used. For determination of dry matter yield and tissue concentration of mineral nutrient element, dry material was used.

**DRY MATTER YIELD:**

Dry matter yield was determined by drying and finely chopped and mixed plants samples in a forced draught oven at 65°C for 24 hours to constant weight. The samples were taken out from the oven and placed in a desiccators, cooled for about an hours and weighed for the determination of yield. Since the dry matter was required for the estimation of different nutrient elements, fresh matter kept for drying was thoroughly cleaned against any surface contamination by first washing with running tap water, rinsing with distilled water and absorbing surface water with clean white blotting sheets. The result for dry matter yield have been expressed in gm / plants

**CHLOROPHYLL:**

Chlorophyll was determined by the method of Petering et al. (1940). Finely chopped and weighed 200 mg of leaf lamina was ground in a pestle and mortar with a little acid washed while silica sand in about 10ml of 80% acetone. The acetone extract was filtered through Whatman No. 42 filter paper in buchner funnel. The residue on the filter paper was thoroughly leached with 80% acetone to remain the last traces of chlorophyll and leachates were mixed. The extract was made to 25/ml and stored in dark in refrigerator till the measurement of colour intensity. The chlorophyll content was measured by estimating the absorption of the acetone extract in. Elico – CL – 20A – Photo – electric – calorimeter used red filter and referring the reading to the standard
calibration curve prepared by the method of Comer and Zscheile (1942). Result for chlorophyll have been expressed in mg/100g FW.

**CATALASE:**

Catalase was assayed in crude tissue extracts. The fresh plant material was used for the assay of catalase. The fresh material was finely chopped and grind with a little acid washed white silica sand in a chilled pestle and mortar in 0.005 M phosphate buffer pH 7, in the proportion of 1 g plant material to 10 ml of the buffer. Grinding was carried out in an ice-bath. The crude extract was filtered through twofold muslin. The catalase was assayed in the crude tissue extracts with in 3 hours of the preparation of the extracts. During this period the extracts were stored in a refrigerator. Where they were not found to under go appreciable loss in activity of the enzyme assayed.

Catalase was assayed by the permanganate titration method of the Euler and Josephsen (1927). 25 ml of 0.01 N hydrogen peroxide was taken in a flask and stabilised at 25°C in a water bath. To this was added 5 ml of properly diluted enzyme extract. The content were thoroughly mixed and 0.05 ml alequate was immediately drawn in test tube containing 5 ml of 2N- sulphuric acid.

Further aliquats from the reaction mixture were drawn at 3, 6, 9 and 12 minutes. The aliquats were titrated against 0.05N KMnO₄ to determine the hydrogen peroxide decomposed. Monomolecular reaction constant was calculated as

\[ K = \frac{1}{t} \log_{10} \frac{A}{A - X} \]
where ‘t’ is time in minutes. ‘A’ is ml KMnO₄ used at 0 minutes and ‘A-X’ is ml KMnO₄ used at 3, 6, 9 and 12 minutes. ‘K’ value for zero time was obtained by extrapolating the 3, 6, 9 and 12 minutes reading. The results have been expressed as units catalase /g FM. The amounts of crude tissue extract taken for enzyme assay was such that by extrapolation of readings of 3, 6, 9 and 12 minutes the reading obtained for zero time was higher than that at 3 minutes. Care was taken to ensure that the activity of the enzyme in the crude extract was in the range in which the activity was found to be proportional to the enzyme concentration in the extract.

**MINERAL NUTRIENT ELEMENTS:**

The estimation of calcium, potassium, magnesium phosphorus, sulphur, iron and manganese were done in oven dry material. The dry matter was thoroughly crushed in a corning glass pestle and mortar and 0.5 to 1.0 g of the dry material was digested with nitric-perchloric acid (Piper, 1942) using 20 ml of nitric acid and 2 ml of perchloric acid per g. of dry matter. The digestion was carried out to incipient drying stage in 100 ml corning glass Erlenmeyer flasks on electric hot plate in a fume chamber. The digest was boiled with 20 ml of glass distilled water for 5 minutes and then made to a suitable volume. For different macro and micro nutrient, estimation were carried out in aliquot drawn from a single digest.

For the estimation of calcium, potassium magnesium, phosphorus and sulphur manesty still distilled water was used and estimations were carried out in borosill and sigcol glass were 1:1 hot HCl washed and distilled water rinsed glass were used.
For iron and manganese, glass distilled water was used and all estimations were carried out in cleaning glassware, the glassware used for estimation as thoroughly cleaned with detergent, rinsed several times with 1:1 hot HCl, distilled water and finally with glass distilled water.

Grease applied to stop corks of burettes was also purified by boiling in glass distilled water.

Calcium, phosphorus and potassium were determined by the method used by D.J.D. Nicholas at Long Aston Research Station, Bristol and described by Wallace (1951).

**CALCIUM:**

The turbidity produced under standardised condition in the case of calcium was measured in Elico CL – 20A photo electric calorimeter using blue filter. The turbidity was measured with in half an hour of the reaction.

**PHOSPHORUS:**

Phosphorus was determined by the molybdenum reduced phosphomolybdic acid blue colour method in \( \text{H}_2\text{SO}_4 \) system. The reductance employed being hydroquinone and sulphide. The blue colour was determined in Elico CL-20A photo electric calorimeter using red filter. The colour measurement was made in half an hour of its development.
SULPHUR:

Sulphur was determined turbidimetrically with barium sulphate by an adaptation of the method of Chesnin and Yein (1951) described by Jackson (1958), the turbidity was measured in Elico CL-20A Photo electric calorimeter using violet filter with in half an hours.

IRON:

Iron was determined calorimetrically as ferrous orthophenanthroline complex by the method described by Humphries (1956). The colour was determined in Elico CL-20A Photo electric calorimeter using blue green filter.

MANGANESE:

Manganese was determined by 'tetrabase' (pp - tetra - ethyl - di - amino - diphenyl - methane) method described by Nicholas and Fischer (1950). The colour measurement was made immediately in Elico CL-20A photo – electric calorimeter using green filter.

NITROGEN:

Nitrogen was determined by semi-micro-kjeldahl method. For estimation of nitrogen, 100 mg dry matter was treated in cold for 2 hours with 2% salicylic acid in H_2SO_4. The salicylic acid was removed by heating with powdered sodium thiosulphate. The sample was then digested by the method of Chibnall et al. (1943). 3 ml. of Nitrogen free sulphuric acid and 0.2 g of catalyst, prepared by grinding Potassium sulphate, Copper sulphate and Sodium selenate was added. The digestion was carried out on an electric hot plate until the digest had
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become blue. The digest was made to a suitable volume. The ammonia produced was distilled by steam distillation in Markhom apparatus into boric acid buffer in an ammonia free atmosphere and estimated by titration with N/140 sulphuric acid containing conway ‘O’ malley indicator (Conway ‘O’ and Malley, 1942).

PRESENTATION OF RESULTS:

The ascorbic acid and chlorophyll contents and catalase and peroxidase activities in plants have been expressed on the fresh weight basis. The yield and macro and micro nutrient elements contents of plants have been expressed on the dry weight basis. The macro nutrient elements have been expressed as percentage dry matter and iron and manganese as ppm dry matter.

The mean values of the blocks A, B and C together with least significant difference (L.S.D.) at P = 0.05 and P = 0.01 have been presented in figures and tables.

STATISTICAL ANALYSIS:

Data were subjected to the analysis of variance according to Steel and Torrie (1960). Entire data have been statistically analyzed with kind suggestions of Mr. Manish Saxena, MCA Deptt., F.G. Institute of Technology, Raibarely, U.P. and tested for significance at 5% and 1% probability levels. The significance of the differences between control and each treatment was determined using the value of least significant different