CHAPTER 2

MATERIALS AND METHODS

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2.1 THE MATERIALS

2.1.1 *Capsicum annuum* L.

Capsicum annuum L. and its variety Ujwala (Plate,1; Fig.,1), Jwalamukhi (Plate,1; Fig.,2), Jwalasakhi (Plate,1; Fig.,3) and Wild (Plate,1; Fig., 4) form the test material. The seeds were brought from the sales counter of Kerala Agricultural University, *Vellanikkara*. The collected seeds were multiplied in the field along with a preliminary observation of different growth traits. Similar sized, full seeds were selected for the treatment.

The treatments were carried out in an area demarked by netting and all the treatments were in triplicates (Plate,1; Fig.,5).

2.1.2 The effluent

Treated effluent from the Ammonia plant of the fertilizer factory, The Fertilizers And Chemicals Travancore Ltd. (FACT), at *Udyogamandal, Aluva* in *Kerala* was collected from the outlet. The collected effluent was assigned as 100 percent. Different dilutions of the effluent like 25, 50 and 75 percent were prepared using distilled water.

2.2 THE METHODS

2.2.1 Treatment with effluent

The seeds were treated with different dilutions of the effluent (25, 50, 75 and 100 percent) for 24 hrs. 100 seeds were treated in each concentration and 60 of them were placed in petridishes lined with moist

filter paper and kept in laboratory conditions. The rest were sown in pots filled with potting mixture prepared in the composition of cow dung, sand, soil in the ratio 1:1:1. Moisture in the pot was maintained by adding the respective concentration of effluent. One control without treatment was maintained for comparison. Three replications were carefully maintained for all experiments.

2.2.2 Evaluation of physico-chemical parameters of the effluent

Analysis of physico-chemical parameters of the effluent that are essential to characterize the quality were carried out according to the standard methods in APHA, 1981.

2.3 PARAMETERS SELECTED FOR THE STUDY

2.3.1 GERMINATION STUDIES

1. Germination percentage

Protrusion of radicle as well as emergence of cotyledons through the seed coat were taken as the criteria for germination. Percentage of germination was expressed on the basis of the total number of seeds tried for germination (Vilasini, 1978).

Seeds showing the signs of germination were counted in each treatment and control and percentage was estimated as:

Germination percentage = (Number of seeds germinated x 100) / Total number of seeds placed.

2. The length of the radicle and hypocotyl

The length of radicle and hypocotyl was measured in centimeters using a thread and scale.

3. Vigour index

The method described by Sharma and Saran (1992) was adopted for determining the vigour index. It was calculated using the formula:

Vigour index = Germination percentage X axis length.

4. Phytotoxicity

The percent phytotoxicity was calculated following Chou and Muller (1972).

$$\text{Percent phytotoxicity} = \frac{\text{Radicle length of control - Radicle length of test}}{\text{Radicle length of control}} \times 100$$

2.3.2 MORPHOLOGICAL STUDIES

1. Height of the plant

The height of randomly selected plants was measured from each treatment and tabulated. Height was measured on 30th, 60th and 90th day after sowing.

2. Number of leaves

The number of leaves on 30th and 60th day after sowing was scored from plants selected at random from each treatment.

3. Number of branches

Number of branches was counted from plants at random from each treatment on 30th, 60th and 90th day after sowing.

4. Floral morphology and reproductive biology

Variation in the morphological characteristics of treated as well as control flowers was noted. They included number of days taken to flower, arrangement and fusion of floral parts and their abnormalities.

5. Root length

Root length was evaluated at harvest from plants selected at random from each treatment and control.

6. Fruit and seed characteristics

The fruit characteristics noted were the number of fruits per plant, weight of fruits, length of fruits and number of seeds per fruit. These were measured from randomly selected 10 samples from each treatment.

2.3.3 CYTOLOGICAL STUDIES

Control and treated seeds were allowed to germinate in moist filter paper lined petridishes. After germination 1cm long root tips were cut and fixed in Carnoy's fluid for 24 hours and kept in refrigerator. Mitotic variations after treatment with the effluent were studied from the semi permanent mitotic squash preparations using acetocarmine. Five slides were prepared for each treatment and three random fields were scored from each slide. Observations were taken as to the number of dividing cells and abnormal cells from each stage of mitosis and compared with the control.

2.3.4 QUANTITATIVE ANALYSIS

The quantitative estimation of the metabolites in leaves was made through spectrophotometric methods.

1.Photosynthetic pigments: Chlorophyll and Carotenoid

The chlorophyll and carotenoid contents were quantitatively estimated by Arnon's (1949) method. The results thus obtained were compared with the control.

A preweighed (250mg) quantity of fresh leaf material was ground into a fine paste. 10ml of 80% acetone was added in to it. The extract was centrifuged and the green supernatant was obtained. Using small quantities of acetone the extract was centrifuged repeatedly till the lechate became colourless. The supernatant was taken together and was made up to 25ml with 80% acetone. The extract was kept away from direct sunlight. The optical density of the extract was read at 480, 510, 645, 652 and 663 wave lengths using spectrophotometer. The samples were analysed in duplicates.

From the optical densities, the chlorophyll and carotenoid contents were calculated using the formula:

Chlorophyll a (mg/gm) = 12.7(OD 663) – 2.69(OD 645) X v/1000xw

Chlorophyll b (mg/gm) = 22.9 (OD 645) – 4.68 (OD 663) X v/1000xw

Total chlorophyll (mg/gm)= OD 652 X 1000/34.5Xv/1000Xw

Carotenoid mg/g= 7.6 (OD 480) – 1.49 (OD 510) X v/1000 X w

Where OD= Optical density

V = Final vol. of 80% acetone (25ml)

W = Wt. of sample taken (0.25gm)

2. Total Carbohydrate

For the quantitative estimation of carbohydrate the method adopted was that of Shirlaw and Gilchrist (1967). The method is based on spectrophotometric observation.

1gm each of Anthron and Thiourea was taken. 760ml of conc. H_2SO_4 was added to 240ml of distilled water. Anthrone and Thiourea were dissolved in this.

200mg of oven dried and ground leaf sample was taken and was boiled for ½ an hour with 20ml of distilled water in a 250ml conical flask. It was filtered and volume was made upto 50ml with distilled water. 1ml of the filtrate was pipetted in Calorimetric tube and 10ml of Anthron reagent was added. After stoppering the tube with rubber plugs they were kept in water bath at 100^0C for 20 minutes. Tubes were removed from water bath and were cooled in running water. The colour density developed was measured spectrophotometrically at 625 nm.

The following equation was used for the calculation:

Mg.Carbohydrate/100gm of the sample = Xa x 20 x 500/reading of the standard OD (2ml of 100 ppm glucose)

Where Xa is the optical density of the sample.

Preparation of standard carbohydrate solution

A standard carbohydrate solution of 100ppm concentration was prepared by dissolving 100mg. glucose in distilled water and making it upto 1000 ml.

3. Total protein

The oven dried leaf sample was ground finely and 50 mg of leaf powder was weighed out. From which further extraction was made using 10 ml of 0.1M NaOH for 12 hrs followed by an additional extraction for 3 hrs. The supernatant obtained after centrifugation was mixed with 20 ml of 10% TCA. It was allowed to stand for 12 hrs. TCA precipitated proteins were removed by centrifugation and was assayed by Lowry's method (Lowry *et al.,* 1951).

Calculation was made using the formula:

$$\text{Concentration unknown} = \frac{\text{OD unknown}}{\text{OD standard}} \times \text{Concentration of standard}$$

Reagent for Lowry Method

Reagent A -2% Na_2CO_3 in 0.1 N NaOH.

Reagent B -0.5% $CuSO_4$ $5H_2O$ in 1% sodium or potassium tartarate.

Reagent C -Mixed 50ml of Reagent A with 1ml of Reagent B

Reagent D -1 part phenol reagent +2 parts water.

0.5ml of protein sample was combined with 5.0ml of reagent C and mixed well. It was allowed to stand for 10 minutes at room temperature. Rapidly added 0.5ml reagent D and mixed immediately and allowed to stand at room temperature for 10 minuets. Read at 600nm, in the spectrophotometer. Bovine serum Albumin was used as the standard.

2.3.5 FOLIAR EPIDERMAL STUDIES

1. Stomatal index

Leaf peels were taken from plants of comparable age for the study of stomatal index.

$$\text{Stomatal index} = \frac{S}{S+E} \times 100$$

Where S is the number of stomata, and E is the number of epidermal cells/unit area.

2.3.6 PHYTOMASS AND PRODUCTIVITY

2.3.6.a Phytomass

Plants were cleaned carefully with water, plant parts were separated and kept in paper packets. They were dried thoroughly in an oven at 80^0 C till the weight became constant. The weight of each plant part was found separately and from this the total phytomass was calculated. The values were compared with those of control.

2.3.6.b Productivity

The net primary productivity was found out by dividing the phytomass value by the age of plant expressed in days (gm/plant/day).

2.3.7 Yield studies

All the plants were carefully observed till harvest. The following yield parameters were noted and tabulated:

2.3.7.1 Date of flowering

The date of first flowering was noted from 3 plants from each treatment and control.

2.3.7.2 Number of fruits per plant

The number of fruits per plant was noted from 3 plants from each treatment and control.

2.3.7.3 Number of seeds per fruit

The number of seeds per fruit was noted from 3 fruits from three plants selected at random from each treatment and control.

2.3.7.4 Fruit weight

The weight of the fruits was taken from three plants from each treatment and control.

2.3.7.5 Fruit Length

The length of the fruit was taken from three plants from each treatment and control.

2.3.8 SCANNING ELECTRON MICROSCOPIC STUDY (SEM STUDY)

In order to study the abnormalities brought about by the effluent on the leaf, pollen and seeds, SEM study was adopted. The specimens were mounted on specimen stubs using double sided sticky tape, coated with gold in vaccum coater and viewed with Hitachi-S-540- stereo scan and photographed.

2.3.9 POLLEN ACETOLYSIS

Pollen preparations were made by the acetolysis method proposed by Erdtman (1952). The materials with the alcohol was transferred to a centrifuge tube. The materials were crushed by a clean glass rod. The

dispersion was passed through a metal sieve and collected in a centrifuge tube. The dispersion was centrifuged and decanted off the alcohol. The sediment was washed with glacial acetic acid. It was then centrifuged and decanted off the acid. 5ml of freshly prepared "acetolysis mixture" (9:1 acetic anhydride and concentrated sulphuric acid) was added to it, the tube was placed with the acetolysis mixture in a water bath at 70^0C and boiled till it attained medium brown colour. Then it was centrifuged, and decanted off the mixture. Glacial acetic acid was added, centrifuged and the acid was decanted. The sediment was washed 3-4 times with distilled water. Again it was centrifuged, the water decanted and a few drops of dilute glycerin was added. It was kept aside for slide preparation.

2.4 STATISTICAL ANALYSIS

The data were analysed statistically using proper statistical tools like percentage, mean, ANOVA (Two-factor with replication) to make a significant conclusion.

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