2. MATERIALS AND METHODS

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In the present investigation an attempt has been made to assess the effects of effluent from Hindustan Newsprint Limited Velloor, on four economically important plants namely Abelmoschus esculentus, (L.) Moench, Cucumis sativus, L.,Lycopersicum esculentum, Mill. and Sesamum indicum, L.. The factory is situated on the banks of Muvattupuzha river. The effluent samples were collected in plastic cans from the main discharge pump and stored in refrigerator. The samples were analysed for various physico-chemical factors (APHA, AWWA and WPCF 1981).

Four concentrations (25%, 50%, 75% and 100%) of the effluent were used for the study. For control the seeds were grown in distilled water. The terms, 'treated and control' were used for polluted and unpolluted plants.

The seeds for the study were sterilized with commercial bleaching preparation-clorax (containing 5.25% sodium hypochlorite) diluted with water in the ratio 1:9 (Hartmann and Kester 1976).

The following parameters were studied in all the four plants.

I. GERMINATION STUDIES

a. Germination percentage

b. Radicle and hypocotyl length

II. COTYLEDONARY STUDIES

a. Stomatal index and stomatal frequency

b. Chlorophyll and carotenoid content

III. MORPHOLOGICAL STUDIES

a. Length of the stem

b. Length of the root

c. Number of leaves

IV. EPIDERMAL STUDIES-MATURE LEAVES

a. Stomatal index and stomatal frequency

V. PHYTOMASS AND PRODUCTIVITY

a. Phytomass-stem

b. Phytomass-root

c. Phytomass-leaves

d. Total phytomass

e. Net primary productivity

VI. YIELD STUDY

a. Number of flowers

b. Number of fruits

c. Weight of the fruits

VII. SEM STUDIES-SEEDS

VIII. HISTOCHEMICAL STUDIES-MATURE LEAVES

a. Qualitative analysis

1. Insoluble polysaccharides - Periodic acid - Schiff's reaction (Mc Cully 1966, Feder and O'Brien 1968).

2. Sulphated and carboxylated polysaccharides - Toluidine blue 'O' Method (O'Brien et al. 1964, Mc Cully 1966, Feder and Wolf 1965).

3. Starch - Iodine potassium iodide reaction (Johansen 1940)

4. Total proteins - Mercuric Bromophenol Blue (Mazia et al. 1953, Ruthmann 1970, Chapman 1975)

b. Quantitative analysis

1. Chlorophyll and carotenoid content

2. Carbohydrate content

3. Protein content

I. GERMINATION STUDIES

Germination studies were conducted under lab conditions. The sterilized seeds were soaked in different concentrations (25%, 50%, 75% and 100%) of the effluent for 12 hrs. For the control, the seeds were soaked in distilled water.

The soaked seeds were kept in petridishes lined with sterilized Whatmann's No.1 filter paper. In each petridish 20 soaked seeds were kept. For each concentration and for the control triplets were kept. The seeds were treated with 2ml of the effluent on alternate days.

a. Germination Percentage

Germination percentage was noted on the first day of germination. Emergence of the radicle was taken as the criteria for seed germination. The number of seeds germinated was noted and the germination percentage was calculated using the following formula:

$$\text{Germination percentage} = \frac{\text{No. of seeds germinated}}{\text{Total No. of seeds}} \times 100$$

b. Radicle and Hypocotyl length

The radicle and hypocotyl lengths were noted on II, III and IV days of germination.

II. COTYLEDONARY STUDIES

The seeds were grown in petridishes for cotyledonary studies. On the V day the observations were made.

a. Stomatal index and stomatal frequency

The peelings of the cotyledons were taken and observed under the microscope. With the aid of prism type camera lucida, the number of epidermal cells and stomata in the field was noted. Stomatal index and frequency were calculated using the following formulae:

Stomatal index = [S/(S+E)] x 100 (Salisbury 1927, 1932)

Where S = Number of Stomata

E = Number of epidermal cells in the field.

Stomatal frequency = $(XY)^2/S \times 10^6$

Where X = Number of stomata in the field.

Y = Magnification

S = Square area of the field

b. Chlorophyll and carotenoid content (Cotyledonary) (Arnon 1949)

For detecting the chlorophyll and carotenoid content, the seeds were grown in petridishes. On the V day of germination, 100mg of fresh cotyledons was accurately weighed out. The material was then ground in a mortar and pestle. Five ml of 80% acetone was added and the cotyledons were thoroughly macerated. The solution was centrifuged until the sedimented material became colourless. The chlorophyll extract was poured into a colorimetric tube and the final volume was made upto 10ml with 80% acetone. The optical density of the extract was read using the colorimeter.

Following formulae were employed to calculate the chlorophyll a, b, total chlorophyll and carotenoid contents.

Chlorophyll-a(mg/gm) = 12.7 (D.663) - 2.69 (D.645) x V/(100xW)

Chlorophyll-b(mg/gm) = 22.9 (D.645) - 4.68 (D.663) x V/(100xW)

Total Chlorophyll (mg/gm) = (D.625 x 1000)/34.5 x V/(1000xW)

Carotenoids = 7.6 (D.480) - 1.49 x D.510 x V/(1000 x W)

Where:

D = Optical density

V = Final Volume of acetone

W = Dry weight of the sample taken

III. MORPHOLOGICAL STUDIES

For the morphological studies the plants were grown in earthenware pots. The sterilized seeds were at first sown on the seed beds. Plants were transplanted to the pot at the two - leaved stage. After 3 days, the treatment was started. The plants were treated with different concentrations (25%, 50%, 75% and 100%) of 200 ml effluent on alternate days. The control plants were watered with distilled water. Triplets were kept for the control and for each treatment. The observations were made during 3 stages of the plant at a time interval of 25 days .

IV. EPIDERMAL STUDIES - MATURE LEAVES

a. Stomatal index and stomatal frequency

Leaf peels were taken and stained in Safranine. Stomatal index and stomatal frequency were found out as in the case of cotyledonary stomata.

V. PHYTOMASS AND PRODUCTIVITY

For determining the phytomass, the plants were cleaned carefully and oven dried at 80°C for 24 hrs. The dry weight of each plant part was taken separately. The total phytomass was also noted. The net primary productivity value (grams per plant per day) was obtained by dividing the phytomass value by the plant age, expressed in number of days.

VI. YIELD STUDY

For the yield, the number of flowers and fruits and the dry weight of the fruits were noted.

VII. SEM STUDIES - SEEDS

Scanning electron microphotographs of the seeds, from the plants grown in 100% effluent and from the control plants were taken using Hitachi S-540 sterioscan on INDU-120 Black and White film.

The negatives were printed on AGFA Professional R-C glossy paper in AGFA MODERN 2-2B enlarger.

VIII. HISTOCHEMICAL STUDIES - MATURE LEAVES

a. Qualitative analysis

The metabolites in the leaf epidermis were localised histochemically. Fresh materials were used for the study. Time, temperature and concentration of each stain were kept constant, inorder to get uniform results. The methodologies of Johansen (1940), Jensen (1962), Krishnamurthy (1988) and Vijayaraghavan and Shukla (1990) were followed.

The metabolites localised and methods used are given below.

1. Insoluble polysaccharides (Periodic acid - Schiff's reaction or PAS Reaction) (Mc Cully 1966, Feder and O'Brien 1968).

The materials were kept in 1% Periodic acid for 20 minutes and then washed in water thoroughly. After placing the

materials in Schiff's reagent for 20 minutes in darkness, they were washed in running water and mounted in glycerine. The purplish red colour developed indicated the presence of insoluble polysaccharides.

2. Sulphated and carboxylated polysaccharides (Toluidine Blue 'O' Method) (O'Brien et al. 1964, Mc Cully 1966, Feder and Wolf 1965).

The material was stained in Toluidine blue 'O' for 5 minutes. Then washed in running water to remove the excess stain and mounted in glycerine.

3. Starch (Iodine - Potassium iodide reaction) (Johansen, 1940)

The material was mounted in Iodine - Potassium iodide solution and watched under the compound microscope.

4. Total proteins (Mercuric Bromophenol Blue Method) (Mazia et al. 1953, Ruthmann 1970, Chapman 1975).

The material was immersed in the dye solution for 15 minutes. Then washed in 0.5% acetic acid for 20 minutes to remove the excess stain. After that washed in water for 20 minutes, and mounted in glycerine.

Colour photographs of the above histochemically localised metabolites were taken.

b. Quantitative analysis

The quantitative analysis of chlorophyll, carotenoid, carbohydrate, and protein content of the leaves was done, in

three stages at a time interval of 25 days. The methodologies followed are as follows.

i. Chlorophyll and carotenoid content (Arnon 1949).

Fresh leaf material was used for the study. Chlorophyll-a, chlorophyll-b, total chlorophyll and carotenoid contents were found out. The method followed is given in detail, where the analysis of cotyledonary chlorophyll and carotenoid content is described.

ii. Carbohydrate content (Anthrone Reagent) (Shirlaw and Gilchrist 1967)

Using an electronic monopan balance, 200mg of dried and milled leaf material was weighed out. This was put into a 250 ml conical flask. To the sample 20 ml of distilled water was added and boiled for 30 minutes. The suspension was then filtered into a 50 ml standard flask and the volume was made upto 50 ml by adding distilled water. Four ml of this filtrate was pipetted into a test tube and to this was added 10 ml of anthrone reagent. The tube was shaken well and closed using cotton plug. The tubes were then kept in the water bath at 100°C for 20 minutes. Then they were taken out and cooled under running water. The colour density developed was measured using a colorimeter at 625 nm wave length.

A standard of 100ppm carbohydrate solution was prepared by dissolving 0.1gm of glucose in 1 litre of distilled water.

The carbohydrate content was calculated using the following formula.

Mg carbohydrate/100gm of sample

= Xax20x500/ Reading of std. OD (2ml of 100ppm carbohydrate)

Xa = Optical density of the sample.

iii. Protein content (Lowry method) (Lowry et al. 1951)

Reagents

Reagent A = 2% Na_2CO_3 in 0.1N NaOH

Reagent B = 0.5% $CuSO_4$. $5H_2O$ in 1% Potassium tartarate.

Reagent C = Mix 50 ml of Reagent A with 1ml of Reagent B (Made fresh each day)

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

50 mg of dried and ground leaf tissue was kept in 10ml of 0.1M NaOH for 12 hrs. for extracting the leaf protein. The solution was centrifuged and the protein extract was obtained. From the extract 0.5ml of protein sample was taken and combined with 5.0 ml of reagent C in a test tube and mixed well. It was kept at room temperature for 10 minutes. 0.5 ml of Reagent D was added to it rapidly and mixed immediately. The mixture was allowed to stand at room temperature for 10 minutes. The colour density so developed was read at 600 mμ in a colorimeter.

The protein content was calculated, employing the following formula.

$$\text{Conc. of unknown sample} = \frac{\text{O D unknown}}{\text{OD std}} \times \text{Conc. of std}$$

The standard solutions were prepared with Bovine Serum. The data were statistically analysed using the Anova technique.