MATERIALS AND METHODS

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A) Material :

Local variety of *Chenopodium album* L. has been selected for the present investigation. Plants were raised in pot soil culture from the seeds of local variety in the botanical garden of Krishna Mahavidyalaya, Rethare BK.The seedlings were allowed to grow and establish under normal conditions with proper irrigation. After 30 days of their normal growth, salt, (NaCl), distillery effluent and vermiwash treatments were given to the plants.

(a) Salinity treatment was given to the plants in increasing concentration of NaCl. The pots were irrigated alternating with tap water and salt water of 4, 8, 12 EC (ms m^{-1}). These four concentrations of NaCl was prepared accordingly to standard procedure described in USDA Handbook-60, control Plants received only tap water. The plants were treated with different concentration of NaCl twice a week with alternating watering the plants with equal amount of water to avoid salt accumulation and also to check the loss of water by evapotranspiraton. 5 treatments of NaCl were given to the plants in triplicate set.

(b) The distillery effluent was collected from the 5^{th} lagoons of Krishna Sahakari Sakhar Karkhana distillery Ltd, Rethare Budruk. The treatment of distillery effluent was given to the plants in increasing the concentration like 10%, 20%, 40%, 60%, 80% and 100% and control was maintained by giving ordinary tap water as irrigants. The two treatments of spent wash were given to the plants after one week of interval.

(c) Vermiwash is a liquid biofertilizer which is applied to the plants in the form of foliar spray. In present investigation vermiwash foliar sprays were given in increasing concentrations like 10%, 20%, 40%, 60%, 80% and 100%. The two treatments of vermiwash spray were given to the *Chenopodium album* after 4 days interval. The irrigation with tap water was given every alternating days.

After 10 days of treatment the plant material was harvested and random samples were taken for the analysis of various organic & inorganic constituent. In the present research work, along with these three treatments, organic and inorganic

constituents were also studied from various stages of leaf developments of *C. album* plant i.e. young. Premature (PM), Mature (M), on set of senescence (O), Senescent stage (S).

B) Methods:

1. Germination Studies:

Germination percentage, seedling growth and vigor index.

Healthy seed of the *Chenopodium album* local variety were selected and they were surface sterilized with 1%, mercuric chloride, then washed with distilled water. Fifty seeds were kept in each sterilizes petridish for germination on Whatman filter paper No.1. The filter paper was moistened with 5 ml of distilled water for control. For distillery effluent treatments six concentrations of effluent like 10%, 20%, 40%, 60%, 80% and 100% were prepared and 5 ml of each concentration is given in different petridishes for filter paper moistered. For vermiwash treatment also six concentration were prepared like 10%, 20%, 40%, 60%, 80% and 100% and applied to 6 different petridishes. In salt treatment, various concentrations of salt (NaCl) solutions viz. EC 4, 8, and 12 $Mscm^{-1}$ were prepared according to the standard procedure described in USDA Handbook- 60.

The emergence of radicle from seed coat was acknowledged as a criterion for germination counts. Experiments were carried out in triplicate at 28^0C in germination chamber under dark. Germination counts were taken after every 24 hours up to 120 hr. stage.

2. Growth Analysis:

The plants after completion of NaCl, distilled effluent & vermiwash treatments were taken for growth analysis Ten plants from each treatment pot were carefully uprooted, thoroughly washed with tap water to remove any dirt and dust particles on the plant parts and then blotted to dry. In the growth analysis only height of the plants was measured to observe the effects.

3. Photosynthetic Pigments:-

a) Chlorophylls:-

Chlorophylls were estimated by Arnon (1949) method. Randomly sampled fresh leaves of different treatment i.e. NaCl, distillery effluent, vermiwash & different stages of leaf development of *C. album. Linn.* were washed with D/W and blotted to dry. 0.5 g plant material were crushed in 80% chilled acetone. A pinch of $MgCo_3$ was added to neutralize the acids released during extraction. The extract was filtered through Whatman No.1 filter paper using Buchner's funnel under suction. Residue was washed repeatedly with 80 % acetone collecting the washings in the same filtrate. Then volume of filtrate was made to 50 ml with 80% acetone. The filtrate was transferred into a conical flask wrapped with black paper to prevent photo-oxidation of the pigments. Absorbance was measured at 663 nm and 645 nm on a UV- VIS double beam spectrophotometer (Shimadzu UV- 190) Using 80% acetone as a blank.

Chlorophylls (mg 100^{-1}g fresh weight) were calculated by using following Formulae –

Chlorophyll 'a' = $[12.7 \times A_{663}] - [2.69 \times A_{645}]$ ---------- **X**

Chlorophyll 'b' = $[22.9 \times A_{645}] - [4.68 \times A_{663}]$ ----------**Y**

Total Chlorophylls (a + b) = $[8.02 \times A_{663}] + [20.2 \times A_{645}]$ ----------**Z**

$$\text{Ch.a/Ch. b/Total Chlorophylls (mg } 100g^{-1} \text{ Fresh weight} = \frac{\text{X/Y/Z x volume of extract x 10}^{\upsilon}}{\text{1000 x weight of plant material (g)}}$$

b) Carotenoids:

Carotenoids were extracted from the weighed amount of leaf material as per the procedure described for chlorophylls earlier. Carotenoids were estimated by the method of Kirk and Allen (1965). The absorbance was recorded at 480 nm on a UV-VIS double beam spectrophotometer (Shimadzu UV - 190). Total carotenoids were estimated using the following formulae -

$$\text{Total Carotenoids (mg 100g-1 Fresh weight)} = \frac{\text{A480 x vol. of extract x 10 x 10}^{\upsilon}}{\text{2500 x Weight of plant material (g)}}$$

Where,

2500 = Average extinction

4. Total Polyphenols:

The total polyphenols in *C. album* treated with NaCl, Vermiwash and distillery effluent as well as different stages of leaf development can be determined by the method of Folin and Denis (1915). 0.5 g fresh leaf tissue were homogenized in 30 ml of 80% acetone and filtered through Buchner's funnel. The residue was washed several times with 80% acetone and the final volume was made 50 ml with 80% acetone. One ml extract along with a series of standard tannic acid (0.1 mg per ml) were taken in separate Nessler's tube and to each tube 10 ml 20% Na_2Co_3 and 2 ml Folin Denis reagent [100 g sodium tungstate mixed with 20g phosphomolybdic acid in about 800 ml D/W to this 200 ml. 25% Phosphoric acid was added and the mixture was refluxed for 2 – 3 hours at room temperature and volume was made to 1000 ml. with distilled water]. The final volume of reaction mixture was made 50 ml with distilled water. After 20 min. absorbance was measured at 660 nm with reagent blank. Total polyphenols were calculated with the help of standard curve of tannic acid and expressed as 100 g^{-1} fresh weight.

5. Carbohydrates:

The sugar was estimated by Nelson (1944) method. The soluble carbohydrates were extracted from 0.5g oven dried powdered leaves of different treatment i.e. salt (NaCl), vermiwash and distillery effluent and also from different stages of healthy leaves i.e. young premature, mature, onset of senescence and senescent leaves. Leaf extracted in 80% neutral alcohol. The extract was filtered through Buchner's funnel using Whatman No.1 filter paper. Then filtrate was condensed on water bath to about 5 ml and 2 gm. Lead acetate and potassium oxalate (1:1) were added for decolorization. Then 40 ml. D/W was added and aliquot was filtered through Buchner's funnel. The volume of filtrate was measured and it served as an extract for determination of reducing sugars. A 20 ml aliquot of this extract was hydrolyzed with 4 ml conc.HCl by autoclaving at the 15 lbs atm. pressure for half an hour. The content was cooled, neutralized with anhydrous sodium carbonate (Na_2Co_3) and filtered. The volume of the filtrate was measured and it was used for estimation of total sugar.

For estimation of starch the insoluble residue along with the filter paper obtained at the beginning after filtering the alcoholic extract was transferred to a 100

ml conical flask with 50 ml D/W and 5 ml conc. HCl and hydrolyzed at 15 lbs atm. pressure for half an hour. These conical flasks were cooled to room temp. neutralized by addition of anhydrous sodium carbonate and filtered through Buchner's funnel using Whatman No.1 filter paper. The volume of the filtrate was measured and this contains reducing sugars (glucose) formed as a result of hydrolysis of starch. Hence it was used for estimation of starch in the residue.

The sugars from all three filtrates were estimated by determining the reducing power by employing arsenomolybdate reagent introducing by Nelson (1944) for the colorimetric determination of the cuprous oxide formed in the oxidation of sugars by alkaline copper tartarate reagent. For this 0.4 ml aliquot (RS and TS) and 0.1ml aliquot for starch were taken in test tube along with different concentration of the standard glucose solution (0.1 mg/ml) in other test tubes. In each test tube requisite amount of distilled water was added to make final volume 1 ml. In case of blank 1 ml D/W was taken instead of filtrate or standard glucose. To this 1 ml somogyi's alkaline copper tartarate reagent (4g.$CuSo_4$, $5H_2O$, 24g unhydraous Na_2Co_3, 16 g Na-K tartarate and 180 g unhydrous Na_2So_4 dissolved in1liter D/W) was added and then the tubes were kept in boiling water bath for 10 minutes. After cooling to room temperature, 1 ml Nelson Arsenomolybdate reagent (25 g Ammonium molybdate dissolved in 450 ml distilled water, 3g sodium arsenate dissolved in 25 ml distilled water, 21 ml concentrated HCl. These ingredients were mixed well and digested for 48 hours at 37^0C) and carefully added. The reaction mixtures were further diluted to 10 ml D/W. The absorbance readings were recorded on a UV-VIS double beam spectrophotometer (Schimadzu UV-190) at 660 nm. The amount of reducing sugars, total sugars and starch were estimated with the help of calibration curve of standard glucose (0.1mg ml^{-1}) and the values were expressed as g 100 g^{-1} dry tissue.

6. Oxalic acid:

Oxalic acid in different stages of leaf development of *C. album* (young, premature, mature, onset of senescence and senescence) were separated into soluble and insoluble fraction as described by Begtsson *et.al.* (1966) and estimated by the method of Abuza *et.al* (1968) for soluble oxalic acid estimation. One gram oven dried plant material and 75 ml double distilled water were taken in a volumetric flask. In another volumetric flask 1 g. oven dried plant material, 10ml 3 N HCl and

65 ml double distilled water were taken for total oxalic acid estimation. Both the flasks were kept for digesting the plant material for 1 hour in boiling water bath. Then flasks were cooled and diluted to 100 ml volume and filtered through Whatman No.1 filter paper. Two aliquotes of 50 ml extract were placed in 150 ml beakers and in each beaker 20 ml 6N HCl added to increase acidity and avoid pectin retention. Then the mixture was evaporated to half volume and filtered through Whatmen filter paper No.1 and ppt was washed several times with warm double distilled water. To this filtrate 3-4 drops of methyl red indicator (1g methyl red in 100 ml alcohol) and then concentrated ammonia solution were added until solution turned faint yellow. Then this solution was heated to 90-100^{0}C carefully on water bath, cooled and filtered to remove interfering ferrous ions containing precipitate.

The filtrate was heated to 90-100^{0}C on water bath and then 10 ml of 5% $CaCl_2$ was immediately added with 20-25 drops of ammonia solution to restore yellow colour. This solution was allowed to settle overnight and on next day it is filtered through Whatman filter paper No.44 (ashless). The precipitate was washed several times with double D/W to make free form Ca [To check whether the ppt is free Ca^{++} 3 ml of washing filtrate was taken in test tube and to it few drops of 5% sodium oxalate was added. The turbidity indicated presence of Ca^{++} and demanded further washing of ppt.]. Then filter paper containing ppt. was dissolved in hot 1:5 H_2So_4 and then diluted to 125 ml with double D/W and transferred to 250 ml conical flask. The content of the conical flask was heated to 90-100^{0}C and carefully titrated with 0.05N $KMnO_4$. The percentage of oxalate was calculated by using following formula-

$$\text{Oxalate } \% = \frac{\text{ml } KMnO_4 \times 0.02 \times 45.02 \times 100}{100 \times \text{dry weight} \times 50 \times 100}$$

7. Inorganic Constituents:

a) Preparation of acid digestion

Inorganic constituents can be estimated by acid digestion method of Toth *et.al.* (1948). The plant material of salt treated, vermiwash, distillery effluent treatments and different stages of leaf development of *C. album* plant i.e young, premature, mature, onset of senescence and senescent stages were carefully washed by tap water and blotted to dryness. The leaves of different treatments, different

concentrations can be separated, packed and subjected to drying in oven at 60^0C for ten days till dried plant material was powdered. 500 mg of oven dried powdered leaf material was transferred to 150 ml capacity beaker to which 20 ml conc. HNo_3 was added. The beakers were covered with watch glass and kept till the primary reaction were completed. Then these beakers were heated slowly to dissolve solid particles. After cooling to room temperature, 10 ml of perchloric acid (60%) were added to it and mixed thoroughly. Then these beakers were heated strongly until a clear and colourless solution (about 2-3 ml) was obtained. It was then cooled and transferred quantitatively to 100 ml capacity volumetric flask, diluted to 100 ml with distilled water and kept overnight. Next day these extracts were filtered through dry Whatman filter paper No. 44 (Ash less.). Filtrates so obtained were used for estimation of different inorganic constituents.

b) Sodium and Potassium:

Sodium and potassium were estimated according to standard flame photometric procedure employing FALCO flame photometer model CL 22A. For standardization various concentrations of Na [20-100ppm] and K[20-100ppm] were prepared from NaCl and KCl respectively. Using these standard solutions standard curves for these elements were obtained. The plant extract (acid digest) was analyzed in the similar manner and values were obtained from the standard curve.

c) Calcium, Magnesium and Iron:

Calcium, Magnesium and Iron were estimated using Atomic Absorption Spectrophotometer (AAS). For standardization various concentrations of Calcium, Magnesium and Iron (20-100ppm) from $CaCl_2$, $MgCl_2$, and Fe_2O_3 respectively were prepared. Standard curve for these elements were prepared. The acid digests of plants were analyzed in the similar manner. If necessary the appropriate dilutions were applied for the analysis.

8. Enzyme Activity:

a) Nitrate Reductase:

Activity of nitrate reductase was determined by the *in vivo* method of Jaworski (1971). The leaf tissue of *C. album* of the different treatments of different conc. were incubated in the medium containing 1ml, 0.1M, KNO_3 , 2ml 5% n-propanol, 5ml 0.2M phosphate buffer pH 7.5 and 2ml 0.5% Triton –X-100 for 1hour

in dark under anaerobic conditions. After one hr. 1ml of reaction mixture was taken out and mixed with one ml of 1% sulfanilamide in 1NHCl and 1ml 0.02 % NEEDA [N-1-naphthyl Ethylene-diamine, Dihydro- chloride], while mixture of 1 ml incubation medium, 1 ml sulfanilamide and 1 ml NEEDA served as a blank. The absorbance was taken at 540 nm on UV-VIS double beam spectrophotometer [Shimadzu 190]. Standard curve was prepared with the help of different concentration of KNo_2 and enzyme activity is expressed as μ g of No_2 liberated h^{-1} g^{-1} fresh tissue.

b) Catalase:

Catalase activity was estimated by following method of Luck (1974) as described by Sadasivam and Manikam (1992). The leaves of *C. album* L treated with NaCl, varmiwash and different stages of leaf of control plant were washed with tap water and blotted to dry. Take 500 mg of plant material and homogenized in 10 ml ice cold (1/15M) phosphate buffer (p^H – 6.8) and filtered through 4 layered musclin cloth. The filtrate was centrifuged at 10,000 rpm for 20 minutes and supernatant was used as source of enzyme. The reaction mixture contained 3 ml of 0.05 ml H_2O_2 in 100 ml phosphate buffer P^H – 7 and 0.1 ml enzyme extract, mixed well and change in OD was recorded at 240 nm. The sloluble proteins in the enzyme extract were determined according to the method of Lowry *et.al.* (1951). The enzyme activity is expressed as unit min^{-1} mg^{-1} protein as described by Bergmeyer (1974).