CHAPTER- II

MATERIAL AND METHODS

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Material :-

2.1 Selection and collection of earthworm species :—

The species of earthworm selected for the present investigation is *Eisenia foetida*. This species is highly adaptive, widely distributed, voracious surface feeding earthworm (Hartenstein *et al* ., 1979, Ismail,1993., Kale, 1997, Ghosh *et al* ., 1999).

The earthworm species *Eisenia foetida* for the present investigation were collected from Department of Vermiculture and vermicomposting, Zilla Parishad, Kolhapur. They were brought to laboratory by using plastic container containing normal soil mixed with buffalo dung.

2.2 Selection of study area and collection of soil samples :—

The study area selected for the present investigation is village Kasabe Digraj of Miraj Tahasil of Sangli district shown in plate No. 1 . fig-1 to 3.

After the selection of the study area, all the three soil samples viz, total saline soil, semi-saline soil and normal soils were collected from

Panbudi area of Kasabe Digraj . The total saline -soil is considered on the basis of barren or non-productive land. Semi-saline soil is one where there is less vegetation or having poor productivity and the normal soil is one which is collected from nearby area where soil is productive and showing full of crops shown in plate No. 1 figs. 1 to 3.

- **Plastic troughs :—**

Ten plastic troughs, having size of about 53.34 cm in length, 22.85 cm in width and 17.14 cm in height were arranged serially. These troughs are shown in plate No. 3 figs-2 and 3. Out of these ten troughs one was used to store earthworm stock and another one was used for acclimatization. The remaining eight troughs were labeled as A1, A2, B1, B2, C1, C2, D1 and D2. and were categorized according to estimated substrate soil samples which is discussed in separate part of this topic.

- **Miscellaneous :—**

For proper handling of earthworms the surgical hand gloves were used. The blunt spatula as well as a sieve having mesh size 2 mm × 2 mm for separating finer soil particles was used.

Plate No.- 3 , Fig. 1 to 3

(Mature adult worm and troughs containing different substrate soil samples and introduction of earthworms for acclimatization)

Fig.1. Showing mature adult earthworm *Eisenia Foetida.*

Fig.2. Showing different troughs containing total saline soil, semi saline soil, normal soil and buffelow dung.

Fig.3. Showing introduction of earthworm *Eisenia Foetida* for acclimation at laboratory condition.

PLATE NO.3

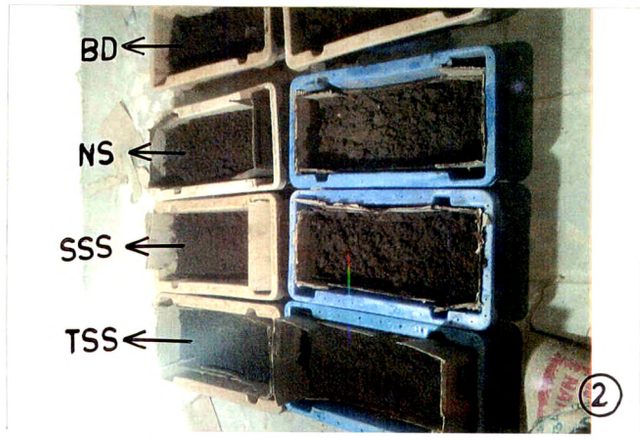


Plate No.- 2 , Fig- 1 to 3

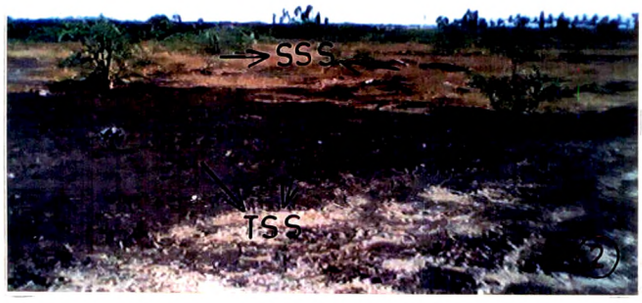
(Different study areas of Shirol Tahasil)

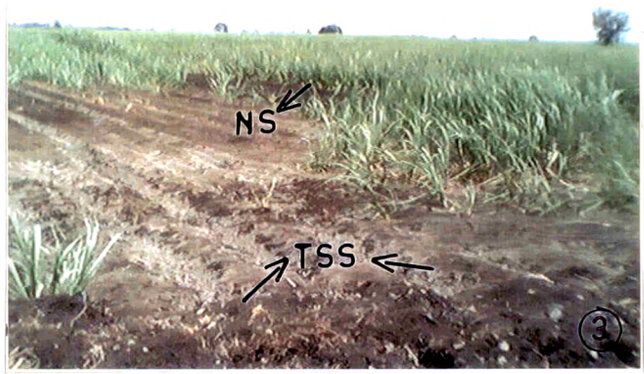
Fig. 1. Study area showing total saline soil of village Shirti of Shirol Tahasil.

Fig. 2. Photograph showing growth of *Parkinsonia aculata* in saline soil of village Shirti of Shirol Tahasil.

Fig. 3. Study area showing total saline soil and productive land with crops at village Shirti.

PLATE NO.2





Sr. No.	BATCH.	No. of Earthworms	Weight in gms.
1.	BATCH-A1	20	6.508
2.	BATCH-A2	20.	6.508
3.	BATCH-B1	20.	6.500
4.	BATCH-B2	20.	6.434
5.	BATCH-C1	20.	6.500
6.	BATCH-C2	20.	6.480
7.	BATCH-D1	20	6.552
8.	BATCH-D2	20.	6.508

Table No. 1 :- Batch preparation of the earthworm

Methods :--

2.3. Stocking of earthworm species :—

The species selected for the present investigation is *Eisenia foetida.* These worms were brought to laboratory and were placed in plastic trough containing normal soil. These worms were acclimatized at laboratory conditions for a week .

2.4. Preparation of earthworm batches :—

A batch of twenty earthworms was selected for introduction in to each set. Prior to introduction into soil samples, the worms were weighed with the help of 'K Roy Classic' one pan balance.

2.5. Methods of soil analysis :—

After collecting the soil samples from study area, the soil samples were analysed by various wel -known and standard analytical methods. The analysis was performed at the beginning of experiment and the readings were recorded.

i) **Testing of physical parameters** :—

1) **Colour** -- The colour of the soil samples was determined by visual estimation.

2) **Temperature** -- The temperature of the soil was measured with the help of soil thermometer. This special thermometer has a long pointed metal arm for insertion in to the deep soil. With the help of this thermometer temperature below the soil was recorded, every week for the entire study period. The mean values for every month during investigation were calculated and recorded.

ii) **Testing of chemical parameters** :—

1) **pH** -- The 5 gms of soil was taken in a beaker. It was passed through sieve having mesh size 2mm × 2mm. It was dissolved in 50 ml distilled water with help of stirrer. The pH was determined on Digital pH meter.

2) **Electrical conductivity** -- The electrical conductivity of the soil after dissolving it in water was measured by Digital conductivity meter (Elico Pvt. Ltd., Hyderabad, India)

PROCEDURE: -- The supernatant liquid of the soil suspension was taken in the specimen tube. Then the conductivity cell was dipped in the

sample and the electrical conductance was recorded by dial reading. The result was expressed in mmhos/cm.

3) Organic carbon and Organic matter --

The organic carbon was determined by Walkey's black method (1947). The values of organic carbon was expressed in per cent and organic matter was calculated by multiplying organic carbon by 1.724., conversion factor.

PROCEDURE:-- 5 grams of soil sample was sieved through sieve having 2mm × 2mm size and was transferred to the conical flask. To this , 10 ml of potassium dichromate and 20 ml of H_2So_4 were added. The flask was kept aside for cooling for about half an hour. After cooling 50 ml of distilled water, 2 ml orthophosphoric acid and 0.5 ml of adiphenylamine indicator were added. It was titrated against ferrous salphate. The blank samples also runned with a set of soil. The organic carbon was calculated by the following formula.

$$\text{Organic carbon \%} = \frac{(S-T) \times N \times 0.003 \times 100}{\text{Mass of soil in grams}}$$

Where, S = Blank reading ,

T = Sample reading ,

N = Normality of potassium dichromate ,

0.003 = Value of organic carbon equal to 1ml, 100 per cent.

The organic matter was calculated by following formula-

Organic matter % = per centage of organic carbon x 1.724.

4) NITROGEN : –

Nitrogen is determined by modified Kjeldahl method

– In 800 ml kjeldahl flask, 5 gms soil sample was taken, digestion catalyst mixture 25 ml of 96 % conc. Sulphuric acid containing 5 % salicylic acid was added and kept it for 30 min.,10 gm sodium sulphate, 0.65 gm Hg and 1 g paraffin added. Afterword the contents are heated first gently and then briskly till contents become clear. There after it was digested for 30 minutes cooled at room temperature.

In the digested sample 200 ml distilled water, some glass beads and few Zinc granules are added. Then the Kjeldahl flask is fitted into the Kjeldahl distillation assembly. 2% of 50 ml boric acid solution containing the mixture indicator is taken in 250 ml Erlenmeyer flask, the tip of

distillation condenser dipped into boric acid solution, feeding funnel of distillation assembly is filled with 10 ml distilled water.

Then 120 ml of 40% Sodium hydroxide solution is added into Kjeldahl flask. Then 50 ml distilled water in it. Thereafter contents of flask are boiled until 120 ml distillate is collected. Similarly a blank determination was run using 0.2 g Sucrose.

Titration of sample and blank distillate collected in 2% boric acid containing mixed indicator. Against standard acid until ammonium borate (NH_4 $B(OH)_4$) is completely neutralized. At the end point the colour of the solution changes from green to distinct pink.

Calculations are made using following formula-

% of N in soil =

$$= \frac{\text{Acid titre (sample-blank) x normality of acid x meq.wt.of N x 100}}{\text{Mass of soil in gms.}}$$

Where,

Meq. Wt of N = 0.014 g

5) PHOSPHORUS :--

The Phosphorus contents was determined by Spectrophotometery by Olsen *et al.,* (1954) method.

PROCIDURE:-

For determination of Phosphrous in soil, 2.5 gms of air dried soil passed through 2 mm sieve, 1 gm activated charcoal free from soluble Phosphrous and 50 ml of 0.5 M $NaHCO_3$ (pH 8.5) into a 250 ml Erlenmeyer flask (conical) then stopper of conical flask is fitted on the mouth and it was shaken by using mechanical shaker at a moderate speed for 30 min. Then the suspension was filtered through Whatman 42 paper collecting the filtrate in a clean receiving flask after discarding 3.5 ml of initial filtrate for determination of Phosphrous concentration in the soil extract, 5 ml aliquot of the sample and blank extract filtrate taken in separate 25 ml volumetric flask. In another 25 ml volumetric flask, 5 ml of 0.5 M $NaHCO_3$ (pH 8.5) solution taken and 5 ml of 1.5% ammonium molybdate solution added in each flask, 1 ml of dilute $SnCl_2$ (freshly prepared) added in each volumetric flask and filled with distilled water.These flask are kept for 10 min. to develop the molybdenum blue

colour. Then the reading of standard and blank measured using incident light at 660 mμ in spectrophotometer.

The Phosphrous in the soil was calculated by using following formula:-

Ppm P in soil =

$$\frac{\mu g\ P/25\ ml\ coloured\ complex\ (\ sample - blank\ )\ x\ volume\ of\ extractant\ used\ for\ preparing\ extract}{ml\ of\ extractant\ taken\ for\ colour\ development\ x\ mass\ of\ soil\ in\ gms.}$$

lbs P/acre = ppm P in soil x 2

$$Kg\ p/ha = \frac{Lbs\ P\ acre\ x\ 2.47}{2.2}$$

Kg P_2O_5 /ha = Kg P/ha x 2.29

6) POTASSIUM :--

The Potassium was determined by Flame photometery—Toth and Prince (1949) .

PROCEDURE:--- 50 g soil was taken and extract was prepared by leaching with 1 N ammonium acetate solution. The reading of sample and

standard solution using potassium filter at 768μ wavelength were recorded on flame photometer and concentration of potassium in the sample was calculated by using following formula:-

Ppm K in soil =

μg K / ml in undiluted extract × volume of extractant used in preparation of extract

(sample – Blank)

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mass of soil in gms

Ibs K / acre = ppm K in soil × 2

Kg K / ha = Ibs K / acre × 2.47 / 2.2

Kg K_2O / ha = Kg K/ha × 1.20

meq K/ 100 soil = Ppm K in soil / Eq. wt of K × 10

7) CALCIUM :--

It was determined by titration method.

PROCEDURE:----- 20 g of air dried soil was taken in a beaker and 250 ml distilled water was added. The suspension was starred and filtered. From this, 10 ml sample was taken in conical flask and 2 ml 1

N NaOH and pinch of murexide indicater were added. These contents were titrated against EDTA solution. The end point was pink to purple. The calcium per centage was calculated by following formula.

Calcium % = A x 400.8 x V/v x 10,000 x S

Where, A = Volume of EDTA used for calcium determination,

V = Total volume of soil extract prepared,

S = Weight of soil,

v = Volume of soil extract titrated (ml).

8) MAGNESIUM:--

It was determined by titration method.

PROCEDURE----- 10 g of air dried soil was taken in a beaker and 250 ml distilled water was added. The solution was starred and filtered 20 ml of filtered sample was taken in a conical flask. 1 ml buffer solution and a pinch of Eriochrome Black –T indicator were added . The contents were titrated against the EDTA solution. The end point of

the experiment was indicated by the change in wine red colour to blue. The magnesium per centage was calculated by using following formula:

$$\text{Magnesium \%} = B - A \times 400.8 \times V/v \times 1.645 \times 10{,}000 \times S$$

Where, A = Volume of EDTA used for Ca ++ determination

B = Volume of EDTA used for Ca ++ and mg ++ ions determination,

V = Total volume of soil extract prepared,

S = Weight of soil,

v = Volume of soil extract titrated.

9) SODIUM :—

Determination of sodium content was done by standard procedure for Flame photometer and concentration of sodium in the sample was calculated by using following formula –

$$\text{Exchangeable Na in soil (meq na/100 g soil)} = \frac{(A-B) \times C \times 100}{10^6 \times D \times E}$$

$$= \frac{(A - B) \times 250 \times 100}{10^6 \times 0.023 \times 10}$$

Where, A and B = ppm Na in sample and blank extract respectively as estimated by reference to standard calibration curve for Na.

C = Total volume of 1N $NH_4C_2O_3O_2$ (pH 7.0) used for the extraction of exchangeable Na from the soil.

D = milligram equivalent weight of sodium.

E = Mass of soil sample in gram taken for the extraction of exchangeable Na from the soil.

10) Micro nutrients of soil – Iron, Manganese, Zinc and copper :-

Soil containing various micro nutrients are measured by Atomic absorption spectrophotometer.

10g of air dried soil (< 2 mm) sample was taken into a 100 ml Erlenmeyer flask. Then 20 ml of 0.005 m DTPA, 0.01 m $CaCl_2$ and 0.1 m Triethanolamine (pH 7.3) added. Then by plugging the stopper contents are shaken on a mechanical shaker at a medium speed. Thereafter the

contents are filtered through funnel lined with Whatman No.42 filter paper. The filtrates are then used for the determination of micronutrient element by the atomic absorption spectrophotometer. Similarly blank is run.

Then the absorbance recorded on atomization of solution of known concentration of an element be noted corresponding to the concentration of the element.

The values of concentrations of an element in sample and blank extract is substituted in the formula

Extractable micronutrient elements in soil (ppm)

$$= \frac{(S-B) \times VE}{M}$$

Where,

S – is ppm concentration of the element in the soil extract as estimated from the recorded absorbance by reference to the element calibration curve.

B – is ppm concentration of the element in the blank test solution estimated from the recorded absorbance by reference to the element calibration curve.

VE – the total volume of the extractant equilibrated with the soil sample.

M – the mass of soil sample in gram taken for equilibration with the extractant with the attachment of computerized reading system. The data recording, processing and print out of the results was simultaneously done.

- **Preparation of sampling sets :—**

Total four double sets of plastic troughs were arranged serially and were labeled as A1, A2, B1, B2, C1, C2, D1 and D2. All these troughs were filled with following types of substrate soil media-

1. Set A1 – buffalo dung
2. Set A2 - buffalo dung
3. Set B1 – normal soil
4. Set B2 – normal soil

5. Set C1 – semi-saline soil

6. Set C2 – semi- saline soil

7. Set D1 -- total saline soil

8. Set D2 – total saline soil

2.6. METHODS OF BEHAVIOURAL STUDIES :—

After releasing the earthworms in to above mentioned sets of various soil samples, the resultant response given by the earthworm *Eisenia foetida* to different substrate soils are recorded in relation to changing their movement like crawling, contact avoidance and try to escape from the troughs.

2.7. METHODS OF MORPHOLOGICAL STUDIES :—

The morphological studies are performed in relation to change in body weight of worms. The weight of worms are recorded in terms of milligrams by using ' K- Roy classic' one pan balance. The growth performance was determined in terms of mean weight gain and relative growth rate (per cent).

Weight gain :- This was taken as the difference between the initial weight of worms stocked and final weight of worms produced.

Weight gain = Initial wt. of worm stocked – Final wt. of worms produced.

Relative Growth rate (RGR) :- This was calculated as per centage ratio of weight gained to the initial body weight as follows –

$$RGR = \frac{\text{Weight gain}}{\text{Initial body weigh}} \times 100$$

2.8. METHODS OF STUDING THE BREEDING OF EARTHWORMS :—

The breeding behavior of earthworm *Eisenia foetida* was studied in relation to mating, cocoon production, hatching success and occurrence of different developmental stages of earthworm in buffalo dung, normal soil, semi-saline soil and total saline soil. The breeding performance was determined as the Net Reproductive Rate, (Nrr) and was computed following Dynes (2003) as stated the following formula –

$$Nrr = \frac{\text{Total No. of earthworms harvested}}{\text{Total No. of earthworms stocked} \times \text{Experimental period (weeks)}}$$

2.9. EXPERIMENTAL PROCEDURE : –

All the sets were kept under supervision for six weeks, proper care was taken during the experiments. The humidity, temperature and moisture content was maintained by proper watering for survival of the earthworm. The limited quantity (50 gms) of cow dung was added in each set. The same procedure was repeated for second time.

The survival rate, growth in the form of weight gain and breeding in relation to cocoon production was recorded.