Materials and Methods

CHAPTER – III

MATERIALS AND METHODS

The details regarding the biodegradation of solid waste (SW) by activated Effective Microorganisms (EM), the pot culture experiment of test plants, to assess the efficacy of EM-SW compost, the various types of treatments involved, the biometric, yield and biochemical parameters recorded and the statistical evaluation of the data are described in this chapter.

3.1 COLLECTION OF MATERIALS

3.1.1 Collection of Solid Waste

Solid waste was collected from the Campus of Avinashilingam University for Women, Coimbatore.

3.1.2 Collection of Effective Microorganisms (EM)

Effective microorganism was collected from Centre for Advanced Studies in Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore.

3.1.3 Collection of Soil

Red soil and sand collected from Maruthamalai area of Coimbatore District was mixed at a ratio of 4:1 (w/w) and used for the pot culture experiment.

3.1.4 Collection of Seeds

The seeds of cow pea [*Vigna ungiculata* (L.) Walp.] and Lady's finger [*Abelmoschus esculentus* (L.) Moench] were bought from Tamil Nadu Agricultural University, Coimbatore.

3.2 MAKING OF BIOMANURE USING EM

3.2.1 Activating the EM

EM is available in a dormant state and requires activation before application. Activation involves the addition of 20 ℓ of water and 2 kg of jaggery (pure cane sugar) to 1 ℓ of dormant EM. The mixture was poured into a clean air tight plastic container and stored at ambient temperatures away from direct sunlight for 8 to 10 days. During the period of activation, a white layer of *Actinomycetes* was formed on the top of the solution accompanied by a pleasant smell and it indicates the activation of EM. The pH is also a determining factor. The pH of the activated EM should be below 4.0.

3.2.2 Solid Waste – Components

Solid waste was collected every day from the campus of Avinashilingam University for Women, Coimbatore (Plate – III).

The non-biodegradable fractions were segregated and discarded and only degradable components were collected for the study. The degradable fractions collected for the study consisted of the following (Table 1 and Figure 1)

PLATE III

Solid Waste Bin Raw
(Avinashilingam University Campus)

Compost Pit with Raw Waste

TABLE – 1

BIO-DEGRADABLE FRACTIONS IN THE COLLECTED RAW SW

S.No.	Components	Percentage of fraction
1.	Paper	5.69
2.	Garden Trimmings and Yard Waste	3.58
3.	Organic Fractions (Vegetables, fruits, foodwaste, etc.)	56.76
4.	Jute	0.92
5.	Wood pieces	1.89
6.	Cloth	4.61
7.	Others	9.32

3.2.3 Compost Preparation

About 20 cm thick SW was put in a clean 3 x 1 x 2 mts composting pit and activated EM was sprayed over this layer. A second layer of SW of about 30 cm. thick was spread over the previous and also sprayed with EM solution. This layering process was repeated to a height of about 100 cm. The stack was completed with a final layer of 5 cm. of cow dung. The entire stack was kept moist by spraying it with activated EM solution at regular intervals. After about 25 to 30 days, the volume of bed dropped substantially and a sweet smelling white mold appeared on the biomass. At this point, the dark brown colored finished compost was collected and sieved, [Margarita, 2001] (Plate – IV).

The compost pile was turned at weekly intervals using pitch fork. The temperature of the pile was monitored 6 times/week at 3 points using 3' long metal stem thermometer. After turning, the piles were remixed. This process was repeated for 60 days. The moisture content of the pile was maintained in the range of 45-60 ^{o} C to all the piles by spraying activated EM solution.

FIGURE – 1

BIO-DEGRADABLE FRACTIONS IN THE COLLECTED RAW SW

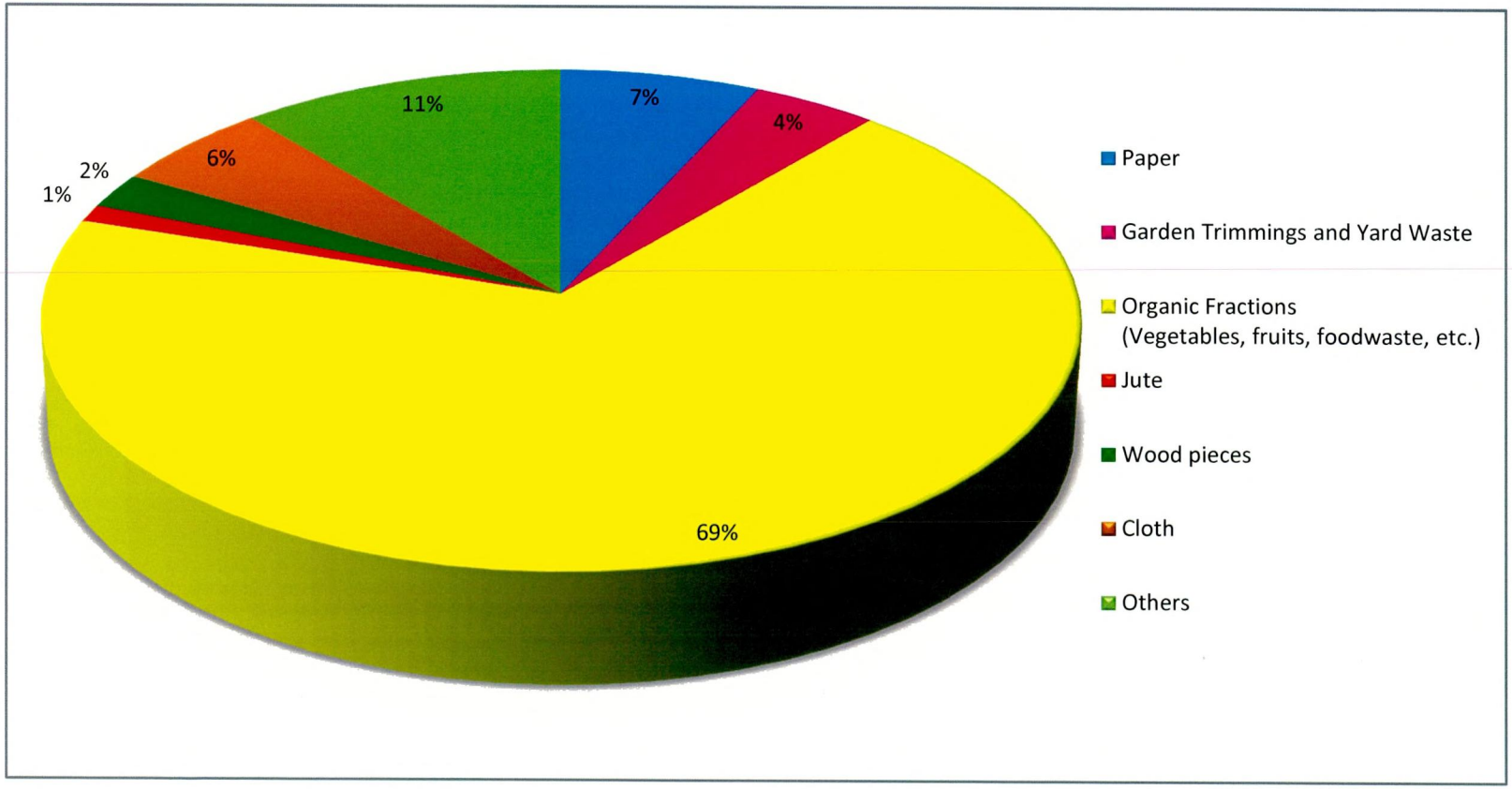

PLATE IV

EM - Solid Waste Compost

Mature Compost 60 Days

3.3 BIOCHEMICAL ANALYSIS OF RAW SW AND EM-SW COMPOST

The following biochemical parameters were analyzed in EM-SW compost at an interval of 15 days for 60 days and were compared with raw SW.

- ❖ Cellulose
- ❖ Organic carbon
- ❖ Total nitrogen
- ❖ Total Phenol
- ❖ Total soluble sugars
- ❖ Reducing and Non Reducing sugars
- ❖ Catalase
- ❖ Peroxidase
- ❖ Dehydrogenase

3.3.1 Estimation of Cellulose (Updegroff, 1969)

Principle

Cellulose undergoes acetolysis with acetic/nitric reagent forming acetylated cello dextrins which got dissolved and hydrolysed to form glucose molecules on treatment with 67 per cent H_2SO_4. This glucose molecule is dehydrated to form hydroxy methyl furfural which forms green coloured product with anthrone and the colour intensity is measured at 630 nm.

Reagents

- Acetic / Nitric reagent: About 150 ml of 80 per cent acetic acid was mixed with 15 ml of concentrated nitric acid.

- Anthrone reagent : 200 mg of anthrone was dissolved in 100 ml concentrated sulphuric acid and chilled for 2 hours before use.
- 67 per cent sulphuric acid.

Procedure

- A quantity of 0.1g of Raw SW and EM – SW compost were taken separately in test tubes, to which 3 ml of acetic/nitric reagent was added and mixed well and kept in a water bath for 30 minutes.
- It was cooled and centrifuged for 15-20 minutes after which the supernatant was discharged.
- The residue was washed with distilled water and 10 ml of 67 per cent sulphuric acid was added and allowed to stand for 1 hour.
- About 1 ml of the solution was taken and diluted to 100 ml.
- From the above diluted solution, 1 ml was taken, to which 10 ml of anthrone reagent was added and kept in a boiling water bath for 10 minutes.
- It was then, cooled and the absorbance was measured at 630 nm.
- A blank was set with anthrone reagent and distilled water.
- The amount of cellulose present in the samples were calculated using a standard graph with 40 to 200 μ g of cellulose.

3.3.2 Estimation of Organic Carbon (Walkley and Black, 1935)

Principle

Organic carbon present in organic matter is oxidized by chromic acid ($K_2Cr_2O_7$) in the presence of concentrated H_2SO_4. This provides nascent oxygen which combines with carbon and form CO_2. The H_2SO_4 enables easy digestion of organic matter by rendering heat of dilution.

Only a certain quantity of chromic acid is used for oxidation. The excess of chromic acid left by organic matter is determined by titration with 0.5 N ferrous sulphate or ferrous ammonium sulphate using diphenylamine indicator.

Reagents

- ❖ Potassium dichromate (1N)
- ❖ Diphenylamine indicator (aqueous)
- ❖ Ferrous sulphate solution (0.5 N)
- ❖ Conc. Sulphuric acid (AR)
- ❖ Orthophosphoric acid (85 per cent).

Procedure

- A quantity of 0.1 g of sieved samples were transferred into a 500 ml conical flasks.
- About 10 ml of 1N potassium dichromate solution was added and stirred well, to which 10 ml of concentrated sulphuric acid was added and kept for 30 minutes.
- Then, 200 ml of distilled water, 10 ml of phosphoric acid and 1 ml of diphenylamine indicator were added.
- The solution in the conical flask was titrated against o.5 N ferrous sulphate and the end point was brilliant bright green.

The organic carbon content in the sample was determined using the formula,

$$\text{Organic carbon (in per cent)} = \frac{(B.V. - S.V.) \times 10 \times 0.003 \times 100}{B.V. \times W}$$

where,

B.V. - Titre value of blank (ml)

S.V - Titre value of sample (ml)

10 - Quantity of 1N potassium dichromate used (ml)

0.003 - One ml of 1N potassium dichromate used (conversion factor)

W - Weight of sample (on dry weight basis)

Organic matter (in per cent) = organic carbon x 1.724.

3.3.3 Estimation of Total Nitrogen

(Microkjeldhal Method – Humphries, 1956)

Principle

A known weight of the powdered sample was treated with diacid mixture so as to oxidize the organic matter and bring the mineral elements into solution.

Reagents

- Diacid mixture : 4:1 (w/w) ratio of conc. Sulphuric acid and conc. Perchloric acid.
- Mixed indicator : 0.5 g bromocresol green and 1g of methyl red were dissolved in 100 ml of 90 per cent ethyl alcohol.
- Sodium hydroxide solution (40 per cent).
- Boric acid (2 per cent).
- Conc. Sulphuric acid : 0.02 N

Procedure

- A quantity of 0.2 g of dried, sieved and homogenized sample was taken in a micro kjeldhal digestion flask (50 ml capacity), to which, 12 ml of diacid was added.
- Complete digestion was ensured by adding one drop of perchloric acid, the contents turned colourless like water.
- The volume was made upto 100 ml with distilled water.
- About 10 ml aliquot was pipetted out into a Wagnor – Parnas distillation apparatus, 10 ml of two per cent boric acid with mixed indicator was kept in a beaker at the delivery end of the distillation apparatus.
- To the distillation apparatus, 10 ml of 40 per cent sodium hydroxide was added and steam distilled. The distillate was collected until no more ammonia was evolved.
- The contents of the beaker were titrated against 0.02N sulphuric acid until a red colour resulted.

Total nitrogen content of sample was determined by the formula.

$$\text{Total Nitrogen (in percent)} = \frac{0.00028 \times T.V. \times 100 \times 100}{10 \times 0.2}$$

where,

Tv = Titre value

0.00028 = One ml of 0.02N Sulphuric acid utilized.

10 = Volume of extract taken for distillation (ml)

0.2 = Weight of sample (g).

100 = Total volume (ml)

3.3.4 Estimation of Phenol

Folin –Ciocalteau Method (Bray and Thorpe, 1954)

Principle

Phenols react with phosphomolybdic acid in folin- ciocalteau reagent in alkaline medium and produce blue coloured complex (molybdenum blue).

Reagents

Sodium carbonate solution – Twenty per cent

Folin-ciocalteau reagent (one volume of this stock solution was diluted with one volume of distilled water).

Ethyl alcohol- Eighty per cent.

Extraction

- A quantity of 20 ml of 80 per cent ethanol was added to 1 g of sample taken in a 100 ml beaker and kept for half an hour at room temperature ($28 \pm 1^{o}C$) after covering it with an aluminium foil.
- It was placed over a boiling water bath for ten minutes.
- The extract was decanted to another beaker.
- The residue was re- extracted with 10 ml of ethyl alcohol and kept over a water bath for 10 minutes.
- The residue was transferred to a mortar and pestle and was ground with 5 ml of eighty per cent ethyl alcohol.
- The residue was filtered through cheese cloth and all the extract were pooled.
- The volume of the extract was made up to nearest whole number with alcohol.

Procedure

- A quantity of 1 ml of sample extract was taken and transferred to a clean test tube.
- To this, 1 ml of folin-ciocalteau reagent and 2 ml of twenty per cent sodium carbonate solution were added.
- The tube was shaken and heated over a boiling water bath exactly for one minute and was cooled under running tap water
- The blue colour developed was diluted with water to 25 ml and its absorbance was measured at 720 nm in a spectrophotometer (uv-vis Spectrophotometer model 108, Systronics, India).
- A blank containing all the reagents without sample extract was used to adjust the absorbance to zero.
- A standard curve was prepared using catechol as phenol, that helped in computing the results.

3.3.5 Estimation of Total Soluble Sugars (Dubois *et al.*, 1956)

Principle

The amount of total soluble sugars present in the extracts can be estimated by anthrone and phenol-sulphuric acid reagents. There is no need to hydrolyse the sample.

Reagent

Anthrone reagent : About 2g of anthrone was dissolved in one litre of conc. H_2SO_4 and prepared fresh.

Procedure

- Aliquots of 1 ml of the extract of sample was pipetted out into test tubes.

- About 4 ml of the anthrone reagent was added.
- A glass marble was placed on the top of each tube to prevent loss of water by evaporation.
- The tubes were placed in a boiling water bath for 10 min.
- The tubes were removed and cooled them to room temperature in a water bath.
- A reagent blank was kept similarly.
- The absorbance of the blue-green solution was measured at 625 nm.
- The amount of sugars present in the extract was calculated using a standard curve prepared from glucose.

3.3.6 Estimation of Reducing Sugars

Dinitroslicylic Acid Method (Miller, 1959)

Principle

Sugars with reducing property (arising out of the presence of a potential aldehyde or keto group) are called reducing sugars. The dinitrosalicylic acid method is one of the classical and widely used methods for the quantitative determination of reducing sugars.

Reagents

Dinitrosalicylic acid reagent (DNS reagent) : 1g Dinitrosalicylic acid + 200mg crystalline phenol + 50 mg sodium sulphite in 100ml of one per cent NaOH.

40 per cent Rochelle salt solution (potassium sodium tartrate).

Procedure

- A quantity of 100 mg of sample was weighed and the sugar was extracted by adding 80 per cent hot ethanol twice (5 ml each time).
- The supernatant was collected and was evaporated by keeping it in a water bath, to which 10 ml of water was added to dissolve the sugars.
- About 0.5 ml of the aliquots were pipetted out into tubes and made up to 3 ml with water, to which 3 ml of DNS reagent was added.
- The contents were heated in a boiling water bath for 5 minutes and 1 ml of Rochelle salt solution was also added in a warm condition.
- After cooling the contents, the intensity was measured at 510 nm.
- The amount of reducing sugars present was calculated using a standard graph.

3.3.7 Estimation of Non Reducing Sugars

The amount of non-reducing sugars present in the sample was calculated by subtracting reducing sugars from total soluble sugars.

3.3.8 Enzyme Assay Preparation for Catalase and Peroxidase

A quantity of 1.0 g of the soil was macerated in 5.0 ml of 0.1 M phosphate buffer pH 6.8 for catalase and 6.5 for peroxidase in a prechilled porcelain mortar and pestle with a pinch of acid washed sand. The homogenate was centrifuged in a refrigerated centrifuge at 10,000 x g for 15 min. The supernatant served as enzyme source.

3.3.9 Estimation of Catalase Activity (Vir and Grewal, 1975)

Principle

More than required amount of H_2O_2 is allowed to react with catalase enzyme for a definite period of time. Remaining unreacted

peroxide is estimated by titrating against 0.01N $KMnO_4$ solution in presence of H_2SO_4. Activity of catalase, is calculated from difference in the amount of peroxide taken initially and the remaining unreacted peroxide.

Reagents

- Hydrogen peroxide (0.3 N)
- Sulphuric acid (2N)
- Phosphate buffer (0.067 M) pH 6.8
- Potassium permanganate (0.01N)

Procedure

- A quantity of 5ml of (0.067 m) sodium phosphate buffer at pH 6.8 was pipetted out into two Erlenmeyer flasks of 100ml capacity.
- 3ml of 0.3N hydrogen peroxide was added to each flask.
- To one of the flasks, 0.1 ml enzyme extract was added and mixed well.
- It was incubated for 15 min at room temperature (28 ± 1°C) with occasional shaking.
- After 15 min, 0.1 ml enzyme extract was added to the second Erlenmeyer flask (control).
- Immediately, 10 ml of 2N sulphuric acid was added to both the flask to arrest the reaction.
- The contents were titrated against 0.01 N potassium permanganate and the titre values were noted.
- End point was the appearance of light pink colour.
- Difference between the titre values (control and test) was the volume of permanganate consumed equivalent to enzyme activity.

- Catalase activity was determined from the volume of 0.01 N Potassium permanganate consumed by the enzyme.

$$\text{Catalase activity (in units)} = \frac{T_2 - T_1}{0.1 \times 15} = Y \text{ ml}$$

where,

T_1 – Volume of 0.01N potassium permanganate utilized by the test solution

T_2 – Volume of 0.01 N potassium permanganate utilized by the control solution

X – $T_2 - T_1$ difference)

0.1 ml – Vol. of enzyme assay

Y = Volume of 0.01 N potassium permanganate utilized by the enzyme min^{-1} mg^{-1} enzyme protein.

Activity of catalase is expressed in enzyme units (U). One unit enzyme activity is defined as equivalent to volume of 0.01 N potassium permanganate utilized $min^{-1}.mg^{-1}$ enzyme protein. Protein content of the enzyme source was determined following the methods of Lowry *et al.* (1951).

3.3.10 Estimation of Peroxidase (Malik and Singh, 1980)

Principle

In presence of hydrogen donor (pyrogallol dianisidine). Peroxidase converts H_2O_2 to H_2O and O_2. The oxidation of pyrogallol or dianisidine to a colour product called purpurogallin is followed calorimetrically.

Reagents

Pyrogallol (0.05 M): A quantity of 630 mg of pyrogallol was dissolved in 100ml of 0.1 m phosphate buffer (pH 6.5) prepared at the time of assay. Hydrogen peroxide - one per cent.

Procedure

- The spectrophotometer (uv - vis 108, Systronics, india) was adjusted to read zero at 430 nm with a cuvette containing 3ml of 0.5 M pyrogallol solution and 0.1ml enzyme extract.
- Immediately, 5 ml of one per cent hydrogen peroxide was added to the test cuvette and mixed well.
- The change in absorbance was recorded for every thirty seconds up to three minutes.
- Protein content of the enzyme source was determined following the method of Lowry *et al.* (1951).
- Enzyme activity was expressed as units. One unit is defined as N moles of pyrogallol oxidased min^{-1}. mg^{-1} enzyme protein at 30°C.

Calculation

Change in absorbance / min at 430 nm = x

Weight of bagasse sample = 1 g

Volume of extract taken for assay = 0.1 ml

Change in absorbance for 0.1 ml of assay = x

Change in absorbance for 5.0 ml = $\frac{x}{0.1} \times 5.0$

Peroxidase activity in g sample = y units min^{-1} mg^{-1} enzyme protein.

3.3.11 Estimation of Dehydrogenase Activity

(Kun and Abood, 1949)

The reduction of nitrate to nitrite may be assayed *in vitro* by incubating the enzyme extract with KNO_2 in presence of an electron donor and *in vivo* by incubating pieces of plant tissues as such in KNO_3 solution. In these methods, the amount of nitrite formed is estimated as a measure of the enzyme activity.

3.3.12 Enzyme Assay Preparation

- The soil sample (0.5 g) was homogenized in a pre-chilled mortar with pestle using a pre-cooled grinding medium consisting of 0.1 M tris, 0.01 M cysteine and EDTA at a pH of 7.0.
- About 4-6 mL of grinding medium was used for g^{-1} tissue.
- The homogenate was filtered through cheese cloth. The filtrate was centrifuged at 20,000 g for 15 min in cold.
- The enzyme assay with the supernatant should be used within 2-3 hr after sampling.

Reagents

0.1 M Potassium phosphate buffer (pH 7.0)

0.1 M KNO_3

1.36×10^{-3} M NADH

One per cent Sulfanilamide in 1.5 N HCl

0.02 per cent N-(1-naphthyl) ethylene diamine hydrochloride

Method

- About 1 mL of potassium phosphate buffer (pH 7.0) was pipetted out into test tubes and 0.2 mL of KNO_3, 0.5 mL of NADH, 0.2 mL of enzyme extract were added along with 0.1 mL of distilled water.
- The tubes were incubated at 30°C for 20 min.
- The reaction was stopped by adding 1 mL of 1 per cent sulfanilamide in 1.5 N HCl and 1 mL of N-(1-napthyl) ethylene diamine hydrochloride.
- The contents were mixed by inverting the tubes and kept for 5 min. to develop the colour.
- It was centrifuged at 1,500 g for 10 min to remove turbidity.
- The controls were maintained without NADH.
- The absorbance of the supernatant was measured at 540 nm in a colorimeter.
- The protein content of the enzyme source was determined following the method of Lowry *et al.* (1951).
- The enzyme activity was expressed as μ moles of TTC formed g^{-1} sample min^{-1} mg^{-1} enzyme protein.

3.4 EFFECT OF EM-SW COMPOST ON TEST PLANTS, LADY'S FINGER [*Abelmoscus esculentus* (L.) Moench.] AND COW PEA [*Vigna ungiculata* (L.) Walp.]

3.4.1 Preparation of the Soil

The soil used for pot culture experiment was air dried, gently ground with wooden mallet, sieved through a two mm sieve and used for the analysis.

3.4.2 Soil Reaction (pH) and Electrical Conductivity

pH and EC (Electrical Conductivity) of the experiment soils were studied using pH meter and electrical conductivity meter in the Department of Botany, Avinashilingam University for Women, Coimbatore.

3.4.3 Pot Culture Experiment

A pot culture experiment was conducted with Lady's finger [*Abelmoscus esculentus* (L.) Moench.] and cow pea [*Vigna ungiculata* (L.) Walp.] as the test plants to evaluate the influence of EM – Solid Waste (SW) compost.

3.4.4 Design and Layout of the Experiment

The design followed for the experiment was a Randomized Block Design consisting of 4 treatments each replicated 3 times.

On the 30 and 60 DAS (Day After Sowing) and at harvest, (90 DAS) various biometric, yield and biochemical parameters observed were evaluated.

3.4.5 Details of Experiment

Test crop : Lady's finger **[*Abelmoschus esculentus* (L.) Moench.]**

Variety : F_1 hybrid.

Test crop : Cow pea (***Vigna ungiculata* (L.) Walp.**)

Variety : Revathi

3.4.6 Treatment details

T_1 - Absolute control

T_2 - EM – SW – compost (20 t ha^{-1})

T_3 - EM – SW – compost (80 t ha^{-1})

T_4 - NPK (N-0.45 g, P- 1.31 g and K- 0.12 g)

The EM – SW compost (20 t ha^{-1} and 80 t ha^{-1}) T_2 and T_3 treatments were compared with standard check, NPK (T_4) and were evaluated against the absolute control, T_1.

3.4.7 Seed Treatment with Rhizobium

The Rhizobium mixed with rice gruel (4g in 10 ml) was coated on the seed surface of leguminous seed, Cow pea. After shade drying, the seeds were used for the experiment.

3.4.8 Cultivation and Treatment Application

Stones and pebbles were removed from the soil and all the pots were filled with 7kg of soil.The size of each pot were 1234545 cu.cm (v/v). About 10 seeds of Lady's finger and Cow pea were sown in each pot and after germination and establishment, seven healthy plants were maintained per pot.

3.5 BIOMETRIC PARAMETERS

3.5.1 Stage 1- 30 DAS (Days After Sowing)

Plant height (cm)

Number of leaves / plant

Root volume (cu. cm)

Number of nodules/ plant

Fresh and dry weight of plant (g)

3.5.2 Stage II – 60 DAS (Days After Sowing)

Plant height (cm)

Number of leaves/ plant

Root volume (cu. cm)

Number of nodules/ plant

Fresh and dry weight of plant (g)

Number of flowers / plant

3.6 YIELD PARAMETERS

3.6.1 Stage III – 90 DAS (Days After Sowing)

Plant height (cm)

Number of leaves / plant

Root volume (cu. cm)

Number of nodules/ plant

Fresh and dry weight of plant (g)

Number of pods / plant

Length of pods (cm)

Single pod weight (g)

Number of seeds / pod

100 seed weight (g)

3.6.2 Root Volume (cu. cm)

To estimate the root volume, a measuring cylinder was taken and water was poured to a certain level and level was carefully noted. The root was immersed fully in water in the measuring cylinder and the rise in the level of water was noted. The difference between the initial and final levels of water represented the volume of the root.

3.6.3 Dry Matter Production

The plant samples including roots collected at 30, 60 DAS and at harvest stages (90 DAS) were air dried and then oven dried at 60°C and the weights were recorded for the dry matter production (DMP).

In all the treatment of test plants, Lady's finger and Cow pea, the following biometric and yield parameters were recorded.

3.7 THE BIOCHEMICAL ANALYSIS OF TEST PLANTS – LADY'S FINGER AND COW PEA

- Total protein
- Total carbohydrates
- Chlorophyll
- Leghaemoglobin
- Ascorbic acid
- Tocopherol
- Catalase
- Peroxidase

3.7.1 Estimation of Protein (Lowry *et. al.*, 1951)

Principle

The blue colour developed by the reduction of the phosphomolybdic phospho tungstic components in the Folin- Ciocalteau reagent by the amino acids tryosine and tryptophan, present in the protein plus the colour developed by the biurel reaction of the protein with the alkaline cupric tartrate are measured in the Lowry's Method.

Reagents

- About 2 per cent of Sodium Carbonate in 0.1N Sodium Hydroxide (Reagent-A)
- 0.5 per cent Copper Sulphate ($CuSO_4$-$5H_2O$) in 1 per cent Potassium Sodium tartrate (Reagent B)
- Alkaline Copper solution : 50 ml of A and 1 ml of B were mixed prior to use (Reagent C)
- Follin – ciocateau Reagent (Reagent D)
- Protein solution (Stock Standard)
- Accurately weighed 50 mg of bovine serum albumin (Fraction V) was dissolved in distilled water and the volume was made up to 50 ml in a standard flask.

Working Standard

About 10 ml of the stock solution was diluted to 50 ml with distilled water in a standard flask. One ml of this solution contained 200 μg protein.

Procedure

Extraction of Protein from Sample

- Extraction is usually carried out with buffers used for the enzyme assay.
- About 50 mg of the weighed sample (leaf) was ground well with a pestle and mortar in 5-10 ml of the buffer and centrifuged.
- The supernatant was used for protein estimation.

Estimation of Protein

- About 0.2, 0.4, 0.6, 0.8 and 1ml of working standard were pipetted into a series of test tubes and 0.1 ml and 0.2 ml of the sample extract in two other test tubes.
- The volume was made up to 1 ml in all the test tubes. A tube with 1 ml of water served as the blank.
- About 5 ml of reagent C was added to each tube including the blank, mixed well and allowed to stand for 10 minutes.
- Then, 0.5 ml of reagent D was added, mixed well and incubated at room temperature in the dark for 30 min. Blue colour developed was read at 660 nm.
- A standard graph was drawn and the amount of protein present in the sample was calculated.

Calculation

The amount of protein present in the sample was expressed in mg g^{-1} or 100 g sample.

3.7.2 Estimation of Chlorophyll Content (Arnon, 1949)

Principle

Chlorophyll was extracted in 80 per cent acetone and the absorption at 663 nm, 645 nm and 652 nm were read in spectrophotometer using the absorption coefficients and the amounts of chlorophyll were calculated.

Reagent

Analytical grade acetone was diluted to 80 per cent (prechilled).

Procedure

- Accurately weighed 1 g of finely cut and well mixed representative sample of leaf was taken.
- About 1 g of leaf tissue was ground to a fine pulp with the addition of 20 ml of 80 percent acetone with a mortar and pestle.
- It was centrifuged (10,000 rpm for 5 minutes)and the supernatant was transferred to a 100 ml volumetric flask.
- The residue was ground with 20 ml of 80 per cent acetone, centrifuged and the supernatant was transferred to the same volumetric flask.
- This procedure was repeated until the residue was colourless. The mortar and pestle was also washed thoroughly with 80 per cent acetone and the clean washing was collected in the volumetric flask.
- The volume was made up to 100 ml with 80 per cent acetone.

- The absorbance of the solution was read at 645, 663 and 652 nm against the solvent (80 per cent acetone) blank.

Calculation

The amount of chlorophyll present in the extract was calculated and expressed in mg chlorophyll g^{-1} tissues using the following equations.

(i) Chlorophyll 'a' mg g^{-1} tissues $= 12.7_{(A\,663)} - 2.69_{(A\,645)} \times \frac{V}{1000 \times W}$

(ii) Chlorophyll 'b' mg g^{-1} tissues $= 22.9_{(A\,645)} - 4.68_{(A\,663)} \times \frac{V}{1000 \times W}$

(iii) Total chlorophyll 'b' mg g^{-1} tissues $= 20.2_{(A\,645)} + 8.0_{(A\,663)} \times \frac{V}{1000 \times W}$

where,

A = Absorbance at specific wave lengths

V = Final volume of chlorophyll extract in 80 per cent acetone

W = Fresh weight of tissue extracted.

3.7.3 Estimation of Carbohydrate

(Anthrone Method - Hedge And Hofreiter., 1962)

Principle

Carbohydrates are first hydrolysed into simple sugars using dilute hydrochloric acid. In hot acidic medium, glucose is dehydrated to hydroxymethyl furfural. This compound forms a green coloured product with anthrone with an absorption maximum at 630 nm.

Reagents

- 2.5N – HCl
- Anthrone reagent : 200 mg anthrone was dissolved in 100ml of ice cold 95 percent H_2SO_4. Prepared, fresh before use.
- Standard glucose: (Stock) – 100 mg of glucose was dissolved in 100 ml water.
- Working standard – 10 ml of stock solution was diluted to 100 ml with distilled water and stored in refrigerator after adding a few drops of toluene.

Procedure

- About 100 mg of the sample(leaf) was taken in a boiling tube with 5 ml of 2.5 N HCl, hydrolysed by keeping it in a boiling water bath for three hours and cooled to room temperature.
- Then, it was neutralized with solid sodium carbonate until the effervescence ceased.
- The volume was made up to 100 ml and centrifuged.
- The supernatant was collected and 0.5 and 1 ml aliquots were taken for analysis.
- From the working standard, the standard was prepared by taking 0, 0.2, 0.4, 0.6, 0.8 and 1 ml and '0' served as blank.
- The volume was made up to 1 ml in all the tubes including the sample test tubes by adding distilled water.

- Then, 4 ml of anthrone reagent was added and heated for eight minutes in a boiling water bath.
- Then, it was cooled rapidly and the green colour developed was read at 630 nm.
- A standard graph was drawn by plotting concentration of the standard on the x-axis versus absorbance on the y-axis.
- From the graph, the amount of carbohydrates present in the sample was calculated.

Calculation

Amount of carbohydrate present in 100 mg of the sample

$$= \frac{\text{mg of gluclose}}{\text{Volume of test sample}} \times 100$$

3.7.4 Leghaemoglobin (Appleby and Bergersen, 1980)

Principle

Haemoglobin reacts with pyridine in strong alkali to produce hemochrome. The hemochrome is measured at 556 nm.

Reagents

- Diluent buffer : 0.1 M Sodium / Potassium phosphate buffer (pH 7.4). Alkaline pyridine reagent : About 0.8 g NaOH was dissolved in 50 ml water and cooled. Then, 33.8 ml of pyridine (33.2g) was added to it, dissolved and diluted to 100ml with water. This produced 4.2 M pyridine in 0.2M NaOH.
- Sodium Dithionite was ground finely and stored in small stoppered tubes in a dessicator.

- Potassium hexacyanoferrate

Procedure

Extraction

- Fresh or thawed nodules were mixed with 1-3 volumes of Phosphate buffer and macerated in a mixer.
- It was filtered through two layers of cheese cloth.
- The nodules debris were discarded.
- The turbid reddish brown filtrate was clarified by centrifugation at 10,000 g for 10 –30 min, and diluted suitably.
- To a suitable volume (2 to 5 ml) of the extract, an equal volume of alkaline pyridine reagent was added and mixed well.
- The solution becomes greenish- yellow due to the formation of ferric hemochrome.
- The hemochrome was taken in equal quantity in two tubes.
- To one portion, few crystals of sodium dithionite was added to reduce the hemochrome and stirred well without aeration.
- The absorbance was measured at 556 nm after 2-5 minutes against a reagent blank in a spectrophotometer.
- To the other portion, a few crystals of potassium hexacyanoferrate was added to oxidize the hemochrome and read at 539 nm in a spectrophotometer after 2-5 minutes against a reagent blank.

Calculation

Lb concentration (mm) = $A_{556} - A_{539} \times 2D / 23.4$

where D is the initial dilution.

(The calculation is based upon the equation $E = 23.4 \times 10^3$ mg^{-1} g^{-1} nodules weight).

3.7.5 Estimation of Ascorbic Acid (Roe and Kuether, 1953)

Volumetric Method

Ascorbic acid, otherwise known as vitamin C is an antiscorbutic. Generally, it is present in all fresh vegetables and fruits. It is a water soluble and heat-labile vitamin.

Principle

Ascorbic acid reduces the 2, 6-dichlorophenol indophenol dye to a colourless leuco-base. The ascorbic acid gets oxidized to dehydroascorbic acid. Though the dye is a blue coloured compound, the end point is the appearance of pink colour. The dye is pink coloured in acid medium. Oxalic acid is used as the titrating medium.

Reagents

- Oxalic acid : 4 per cent
- Dye Solution : About 42 mg sodium bicarbonate was dissolved into a small volume of distilled water along with 52 mg, 2,6-dichloro phenol indophenol in it and the volume was made up to 200 mL with distilled water.
- Stock Standard Solution : 100 mg ascorbic acid was dissolved in 100 mL of 4 per cent oxalic acid solution in a standard flask (1 mg / mL).

- Working Standard : 10 mL of the stock solution was diluted to 100 mL with 4 per cent oxalic acid. The concentration of working standard is 100 μg^{-1} mL^{-1}.

Procedure

- About 5 mL of the working standard solution was pipetted out into a 100 mL conical flask and 10 mL of 4 per cent oxalic acid was added to it. The mixture was titrated against the dye (V_1 ml). End point was the appearance of pink colour which persisted for a few minutes. The amount of the dye consumed was equivalent to the amount of ascorbic acid.

- The extract of the leaf sample (0.5 mg) was taken in 4 per cent oxalic acid and centrifuged. The volume was made upto 100 ml.

- About 5 mL of this supernatant was pipetted out and 10 mL of 4 per cent oxalic acid was added. The mixture was titrated against the dye (V_2 ml).

Calculation

$$= \frac{0.5\ mg}{V_1\ mL} \times \frac{V_2}{5\ mL} \times \frac{100\ mL}{\text{Weight of the sample}} \times 100$$

Amount of ascorbic acid is expressed as mg g^{-1} sample.

3.7.6 Estimation of Tocopherol (Rosenbergh, 1992)

Reagents

- Absolute alcohol.
- Xylene

- 2,2-Diphyridyl : 1.2 g of 2, 2-Diphyridyl in 1000 ml of n-propanol.
- $FeCl_3$ solution : 1.2 g of $FeCl_3$. 6 H_2O in 1000 ml of ethanol and was kept in a brown bottle.
- Standard Solution of D, L-α-Tocopherol : 110 mg of tocopherol acetate was accurately weighed and dissolved in 100 ml alcohol.
- 0.1 N H_2SO_4 : (0.24 ml of H_2SO_4 was made up to 100 ml).

Procedure

- Accurately weighed 2.5 g of leaves were homogenized in a blender taken in a flask (leaf alone is homogenized).
- Then, 50 ml of 0.1 N H_2SO_4 was added slowly without shaking and allowed to stand over night.
- The contents of the flask was shaken next day and filtered through filter papers and the aliquot was taken.
- 3 conical flasks (stoppered) were taken and 1.5 ml leaf extract, 1.5 ml ethanol and 1.5 ml xylene were added in the 1st flask. In the 2nd flask 1.5 ml standard, 1.5 ml H_2O and 1.5 ml xylene were added and in the 3rd flask 1.5 ml H_2O, 1.5 ml ethanol and 1.5 ml xylene were added.
- From the above solution, 1.0 ml of xylene layer was transferred into other tubes without mixing of ethanol.
- About 1 ml of 2, 2-dipyridyl reagent was added in all tubes and mixed.

- Then, 1.5 ml of the mixture was taken, Test, sample, standard and blank were read at 460 nm.
- Then, 0.33 ml of $FeCl_3$ solution was added in all the tubes, mixed well and kept for 15 minutes.
- Then they were read at 520 nm.

Calculations

Amount of tocopherol present ($\mu g\ g^{-1}$)

$$= \frac{(\text{Reading at 520 nm} - \text{Reading at 460 nm}) \times 0.24 \times 15}{\text{Reading of standard at 520 nm}}$$

The activity was expressed at $\mu g\ g^{-1}$ tissue.

3.7.7 Estimation of Urease

Nesslerization Method (Sumner, 1955)

Principle

Urease catalyses the hydrolysis of urea to CO_2 and NH_3. This is then treated with Nessler's reagent and the yellow colour developed was read at 495 nm.

Reagents

- 30 mM urea
- 10 per cent TCA
- Nessler's reagent : About 100 g of mercuric iodide and 70 g of potassium iodide were taken in 1000 ml volumetric flask containing 400 ml of water and stirred well to dissolve the contents.

- About 100 g of NaOH was dissolved in 500 ml water, cooled and added to the mixture in the flask by constant stirring.
- The mixture was diluted to 1000 ml with water.
- The mixture was decanded and used for further experiment.

3.7.8 Enzyme Assay Preparation

- About 0.5 g of soil sample was homogenized in 0.1 M phosphate buffer (pH 7.1).
- The homogenate was centrifuged at 20,000 g for 15 min. and then filtered.
- The supernatant was used as enzyme source.

Procedure

- About 0.5 ml of reaction mixture was taken.
- To this, 0.1 ml of 30 mM urea and 0.1 ml of enzyme extract were added.
- It was incubated for 15 min at 37°C.
- The reaction was stopped by the addition of 0.2 ml of 10 per cent TCA.
- Along with these, controls were also set up, to which, enzyme was added at the end of the incubation period.
- The mixture was allowed to stand for 30 min and centrifuged.
- About 0.5 ml of supernatant from each tube was transferred to other tubes.

- A quantity of 1.0 ml of Nessler's reagent was added and the volume was made upto 10 ml with distilled water.
- The colour developed was read at 495 nm after 5 min.
- The protein content of the enzyme source was determined following the method of Lowry *et al.*(1951)

The urease activity is expressed as μ moles of ammonia formed min^{-1} mg^{-1} enzyme protein.

3.8 STATISTICAL ANALYSIS (Panse and Sukhatme, 1978)

The data obtained from various experimental observations were subjected to the statistical analysis. Based on the results, inferences were drawn. Wherever necessary, the data were analyzed by applying techniques for analysis of variance for completely randomized block design. Treatments were compared using Duncan's Multiple range test.