3. MATERIALS AND METHODS

The present research had been carried out the effect of raw sugar mill effluent and biological treated effluent (BTE) on groundnut (Arachis hypogaea L.)

MATERIALS

Seed and plant materials

Ten varieties of groundnut seeds (Arachis hypogaea L. var. (VRI 2, VRI 3, VRI 4, VRI 6, VRI 7, TMV 2, TMV 7, CO 2, CO 3, and JL 24). were obtained from Oil Seeds Research Station, Regional Research Station of Tamil Nadu Agricultural University located at Virudhachalam and Tindivanam, in Tamil Nadu, India. The healthy seeds were chosen and used for plate, pot and field culture experiments.

Water plant

The aquatic plants such as water hyacinth and water lettuce (Eichhornia crassipes and Pistia stratiotes) were collected from a freshwater pond from Vandigate, Chidambaram and Muthandikuppam, in Tamil nadu, India. The plants of same size and weight were selected and used for the biological treatment of sugar mill effluent.

The raw effluent samples were kept in plastic tubes of 20 litres capacity water hyacinth and water lettuce (Eichhornia crassipes and Pistia stratiotes) were introduced into the effluent. The plants were allowed to remain for two days (48
The mineral present in the effluent which is toxic to the crop was observed by these plants. The treated effluent samples were used for irrigation analysis of the sugar mill effluent.

**Field experiments**

Field experiments were conducted to analyse the different concentration of sugar mill effluent on the tolerant variety of groundnut (VRI - 2). The experiments were conducted during the period from February 2010 to August 2010 at Department of Agriculture, State Oil and seeds farm, Neyveli.

**Field Preparation**

The experimental field was ploughed thoroughly three times before sowing. The entire field was irrigated two days before sowing. The seeds of groundnut were sown at an interval of 20 X 20 cm.

**Details of the experiment**

- **Experimental Period**: February 2010 to August 2010.
- **Tolerant variety used**: VRI 2.
- **Experimental design**: Randomized block design.
- **Plot size**: 1 X 1 m²
- **Treatments**: i) Control
  
  ii) BTE (*Eichhornia crassipes*)
  
  iii) BTE (*Pistia stratiotes*)
- **Replications**: Three
- **Concentrations**: 10, 25, 50 and 100 per centage.
**Irrigation schedule**

The experimental plots which receive 100 per cent well water were treated as control, whereas the BTE plots were received with appropriate amount of sugar mill effluent solutions. As per the experimental schedule BTE contains more nutrients and minerals which were dissolved in water and irrigated in equal volume twice a week till the harvest stage.

**TREATMENT DETAILS**

The treatment details are as follows:

- \( T_1 \) – Control (Untreated)
- \( T_2 \) – 10 per cent effluent
- \( T_3 \) – 25 per cent effluent
- \( T_4 \) – 50 per cent effluent
- \( T_5 \) – 100 per cent effluent

**EFFLUENT SAMPLES**

**Collection of effluent samples**

The sugar mill effluent was collected from the outlet E.I.D Parry sugar factory, Nellikuppam, Cuddalore district, Tamil Nadu. The effluent stored in refrigerator at 5°C to avoid changes in its characteristics, and brought to the Laboratory for the physico chemical analysis purpose, as per the routine standard methods mentioned in American Public Health Association (APHA, 2005).
METHODS

Physico-chemical analyses of sugar mill effluent

Colour

The colour of the effluent was observed visually.

Odour

It was categorized as objectionable or non-objectionable by direct smelling of the sample.

pH

The pH of the effluent sample was measured by pH meter in the field itself.

Electrical conductivity

The electrical conductivity of the effluent sample was measured by using a self-contained conductivity meter at 25°C.

Temperature (°C)

The temperature was measured by using a standard thermometer in the field itself.

Suspended solids

Hundred ml of volume of the sample filtered through the filter paper. The filtrate was washed with distilled water to remove the soluble salts. The crucible was kept in an oven at 103-105°C and the final weight was taken. The amount of suspended solids can be calculated by using the formula.

Total suspended solids = Total solids – Total dissolved solids
**Dissolved solids**

The dissolved solids were estimated by filtering a known volume of 100 ml of well mixed sample through a standard glass fibre filter. The filtrate was evaporated to dryness.

\[
\text{Total dissolved solids (mg/l)} = \frac{\text{Weight of salt in dish}}{\text{Volume of sample}} \times 10^6
\]

**Biological oxygen demand (BOD)**

Biological oxygen demand of the effluent was determined by incubating BOD bottles containing the samples kept at 20°C for five days. The samples were diluted with distilled water and 1 ml of phosphate buffer, magnesium sulphate, calcium chloride and ferric chloride were added to aerate distilled water and the pH was determined. The dissolved oxygen content of one set was estimated immediately following the Winkler’s method of estimation of dissolved oxygen. Another set was incubated for 5 days in BOD incubator and the incubated samples were used for the estimation for dissolved oxygen.

\[
\text{BOD (mg/L)} = \left( \text{Dissolved oxygen before incubation} \right) \times \left( \frac{\text{Dissolved oxygen after incubation}}{\text{Dilution factor}} \right)
\]

**Chemical oxygen demand (COD)**

Ten ml of 0.1 N potassium dichromate, 1 g silver sulphate and mercuric sulphate were added besides 30 ml of concentrated sulphuric acid in a 50 ml COD flask. The mixture was kept for 2 h in a water bath. After cooling, the solution was made up to 140 ml by adding distilled water. Ferroin indicator (0.5 ml) was added and mixed thoroughly. Then, the solution was titrated against 0.1 N ferrous
ammonium sulphate (FAS). Then, the COD of the given sample was calculated by using the formula

\[
\text{COD (mg/l)} = \frac{(a - b) \times \text{normality of FAS} \times 8000}{\text{Volume of sample}}
\]

\[
a = \text{ml FAS used for blank}
\]

\[
b = \text{ml use for sample}
\]

**Dissolved oxygen**

Fifty ml of sample was taken in a 250 ml glass stoppered bottle. One ml of manganese sulphate and one ml of alkaline potassium iodide solution were added to the sample. The bottle was shaken well and the precipitate was allowed to settle. 1ml of conc. sulphuric acid was added to dissolve the precipitate. Five drops of freshly prepared starch indicator was added and then the solution was titrated against standard sodium thiosulphate. The end point was the disappearance of dark blue colour.

**Phosphate**

The phosphate in wastewater reacts with ammonium molybdate and form complex heteropoly acid, which gets reduced to a complex of blue colour in the presence of Zn Cl₂. The absorption of light by this blue colour can be measured at 690 nm to calculate the concentration of phosphates with standard curve.

**Reagents**

**a) Ammonium molybdate solution**

i) 25.0 g of ammonium molybdate was dissolved in 175 ml of distilled water.

ii) 280 ml of concentrated H₂SO₄ was add to 400 ml distilled water and the two solutions (a) and (b) were mixed and diluted to 1 l.
b) **Stannous chloride solution**

2.5 g of stannous chloride was dissolved in 100 ml glycerol by heating on a water bath for rapid dissolution.

c) **Standard phosphate solution**

4.388 g of dried anhydrous potassium phosphate (K$_2$HPO$_4$) was dissolved in distilled water and made the volume to 1 l. This solution was diluted into 100 l. This standard solution contained 10 mg/l.

**Sulphide**

7.5 ml of the effluent sample was taken into two 10 ml comparator tubes (125 mm long and 15 mm outside diameter). The two tubes were marked as A and B. 0.5 ml amine-sulphuric acid added into tube A and then 3 drops ferric chloride solution was added. The sample was thoroughly mixed and blue colour is formed, 0.5 ml of sulphuric acid and 0.15 ml ferric chloride solutions were added into tube B. After three minutes, 1.6 ml diammonium hydrogen phosphate solution was added to each tube to remove the colour of the ferric chloride. They were allowed to stand for 10 min. Methylene blue solution I and II was added to determine the sulphide.

\[
\text{Sulphide (mg/l)} = \frac{\text{ml methylene blue I}}{0.05} = \frac{\text{ml methylene blue II} \times 0.1}{0.05}
\]

**Magnesium**

Fifty ml of effluent sample was taken in 1000 ml conical flask. Twenty ml of ammonium chloride buffer was added. Three drops of Eriochrome blue-T
indicator was added to this solution. It was titrated against EDTA solution. The end point is blue from wine red.

\[
\text{Magnesium (mg/l)} = \frac{\text{Titration value} \times 0.96 \times 1000}{\text{Volume of sample}}
\]

**Sulphate**

Hundred ml of sample was taken in a conical flask. Ten ml concentrated HCl was added and boiled for 5 min. While boiling, 20 ml of barium chloride solution was added and continued the boiling for 5 min more. It was cooled for overnight. The liquid was discarded and filter the precipitate through Whatmann No. 42. It was dried, cooled, weighed and calculated the sulphate present in the given sample.

\[
\text{Sulphate (mg/l)} = \frac{\text{Weight of precipitate} \times 10^6 \times 96}{\text{Volume of sample} \times 233}
\]

**Phenols**

Fifty ml of sample + 10 ml of ammonia-ammonium chloride buffer was taken in 50 ml of aliquot flask. Further 3 ml potassium ferricyanide was added and allowed to stand for 2 min. The colour of the sample was measured, the absorbents at 460 nm in UV-Spectrophotometer against blank.

**Sodium**

Digested sample of the effluent was subjected to determine the potassium content using flame photometer with sodium filter. The potassium content was determined from the calibration curve. Standard potassium chloride solution was prepared and fed into the flame photometer to draw a standard curve and analyzed potassium value.
Sodium could be determined by dividing the percentage of the sodium concentration to the cationic concentration.

\[
\text{Sodium (per cent)} = \frac{100 \times \text{sodium content (mg/L)}}{\text{Na + Ca + Mg + K content (mg/L)}}
\]

**Total nitrogen**

The effluent sample was digested with sulphuric acid containing potassium sulphate and mercuric sulphate as catalyst to convert organic nitrogen to ammonium sulphate. The digested effluent was decanted into the micro-Kjeldahl distillation flask. Along with sample, 10 ml of 40 per cent sodium hydroxide was added and distilled. The distillate was collected in a conical flask containing 10 ml of 4 per cent boric acid and three drops of mixed indicator. The sample was titrated against 0.05 N hydrochloric acids and the nitrogen content was estimated.

**Chloride**

A known volume of effluent sample was taken and pH was adjusted to neutral by adding H₂SO₄. One ml of K₂Cr₂O₄ indicator solution was added. The samples were mixed thoroughly and it was titrated with AgNO₃ solution to form pinkish yellow colour. The chloride content was estimated by using the formula.

\[
\text{Chloride (mg/l)} = \frac{(A - B) \times N \times 3540}{\text{Volume of sample}}
\]

\[
A = \text{ml AgNO₃ solution for sample}
\]

\[
B = \text{ml AgNO₃ for blank}
\]

\[
N = \text{Normality of AgNO₃}
\]
Calcium

Fifty ml of sample was taken in a conical flask. Five ml of triethanolamine and pinch of calcium indicator was added. Hundred ml of distilled water and 10 ml of KOH were added. It was titrated against EDTA solution. End point is the appearance of pink colour from greenish pink.

\[
\text{Calcium (mg/l)} = \frac{\text{Titration value} \times 1.6 \times 1000}{\text{Volume of sample}}
\]

Copper (Neocuproine method)

Fifty ml of aliquot diluted sample was taken and 0.1 ml of conc. HCl was added and boiled. The cooled sample was transferred to a 125 ml separating funnel. The content was diluted with distilled water and 0.1 ml HCl was added in each 5 ml solution of hydroxylamine hydrochloride + 10 ml of sodium citrate and 10 ml of neocuproine solution. The sample was added in each blank and 20 ml of chloroform compound was added. The colour of sample was read at 457 nm in spectrophotometer and compared with standard graph.

Lead (Dithizone method)

Twenty five ml of sample was taken in a conical flask 3 drops of indicator were added to the sample. One drop of diluted nitric acid was added until they change red colour into yellow. Powdered hexamine was added to get red colour. The sample was titrated against EDTA solution until the colour changed from red to yellow.
\[
Pb (\text{mg/l}) = \frac{\text{Titrated value} \times 8.2884 \times 1000}{\text{Volume of sample}}
\]

**Iron**

Hundred ml of sample was taken in 250 ml beaker and 5 ml of 1 N HCl was added. The volume was reduced to 40 ml by using a hot plate. It was cooled and then potassium permanganate solution was added drop by drop until the appearance of pink colour. It was transferred to 50 ml Nessler tube and made up to the mark. 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 ml iron standard solution was pipetted into 50 ml Nessler tubes. One ml of diluted HCl and 2 drops of potassium permanganate solution were added. They were mixed well and made up to the mark with distilled water. One ml of thiocyanate solution was added to the sample and mixed well. The colour of the sample was compared with that of the standard one and find out the amount of iron present in the sample.

\[
\text{Iron as Fe (mg/l)} = \frac{\text{Matching standard} \times 0.01 \times 1000}{\text{Volume of sample}}
\]

**Chromium**

This method was based upon the oxidation of iodide to iodine by dichromate in acidic solution. The liberated iodine was titrated with standard thiosulphate solution. To 100 ml of water sample, 2 ml of 10 N H\textsubscript{2}SO\textsubscript{4}, 1 g ammonium bifluoride and 1 g sulphonic acid were added. They were dissolved it thoroughly and 2 g potassium iodide were added. The dissolved solution was kept in dark for five minutes. The solution was titrated with 0.1 N sodium thiosulphate.

**Calculation**

\[
\text{Chromium(\mu g/l)} = \frac{\text{ml thiosulphate} \times N \text{ of thiosulphate} \times 17.332 \times 1000}{\text{ml sample taken}}
\]
Potassium

Digested sample of the effluent was subjected to determine the potassium content using flame photometer at 769 nm. The potassium content was determined from the calibration curve. Standard potassium chloride solution was prepared and fed into the flame photometer to draw a standard curve and analyzed potassium value.

PREPARATION OF EFFLUENT CONCENTRATIONS

The collected effluent sample from the outlet of sugar mill effluent was considered as 100 per cent raw effluent. Different concentrations (10, 25, 50 and 100 per cent) of sugar mill effluent were prepared freshly by using tap water whenever necessary. They were used for all experiments. For 100 ml,

- Control : Tap water
- 10 per cent : 10 ml effluent + 90 ml of water
- 25 per cent : 25 ml effluent + 75 ml of water
- 50 per cent : 50 ml effluent + 50 ml of water
- 100 per cent : Raw effluent (undiluted)

GERMINATION STUDY:

VARIETAL SCREENING EXPERIMENT

The healthy seeds of groundnut varieties ((VRI 2, VRI 3, VRI 4, VRI 6, VRI 7 TMV 2, TMV 7, CO 2, CO 3, and JL 24)) were surface sterilized with 0.1 per cent mercuric chloride for 2 min and washed thoroughly with tap water and then with distilled water. Twenty seeds of ten varieties of groundnut seeds were arranged equispacially in plastic trays lined with filter paper. They were irrigated
uniformly with equal volumes (10 ml) of different concentrations (10, 25, 50, and 100 per cent) of sugar mill effluent. The seeds irrigated with tap water were treated as control. They were allowed to grow for a week. Three replications were maintained for this varietal screening experiment.

**Germination percentage**

The number of seeds germinated in each treatment was counted on each and every day up to 7th day after sowing. The total germination percentage was calculated by using the following formula:

\[
\text{Germination percentage} = \frac{\text{Total number of seeds germinated}}{\text{Total number of seeds sown}} \times 100
\]

**Seedling length (cm/seedling)**

Twenty seedlings were randomly selected on 7th day from each treatment to record the seedling growth. The growth of the ten varieties of groundnut seedlings was measured by using a centimeter scale and the values were recorded.

**Fresh weight and dry weight (g/seedling)**

Twenty seedlings were taken, air-dried and their fresh weight was taken. The same seedlings were kept in a hot air oven at 80°C for 24 hours. Then, the samples were kept in desiccators and their dry weight was taken by using an electrical single pan balance. The average was expressed in g/seedling.

On the basis of the data obtained from varietal screening experiments, the variety VRI 2 was identified as tolerant when compared with other varieties tested. So, the tolerant variety VRI 2 was taken for further experiments.
EXPERIMENT WITH TOLERANT VARIETY

Germination study was conducted with the tolerant variety VRI - 2 the seeds were equispacially arranged in plastic cup with different concentrations (10, 25, 50 and 100 per cent) of sugar mill effluent. The germination studies parameters such as germination percentage, root length, shoot length; fresh weight and dry weight were taken by following the methods described in varietal screening experiment. From these data, the following values of vigour index, tolerance index and phytotoxicity were also calculated. Three replicates were maintained for this experiment.

Vigour index

Vigour index of the seedlings were calculated by using the formula proposed by (Abdul-Baki and Anderson, 1973).

\[ \text{Vigour index} = \text{Germination percentage} \times \text{seedling length.} \]

Tolerance index

Tolerance index of the seedlings were calculated by using the formula proposed by (Turner and Marshal, 1972).

\[ \text{Tolerance index} = \frac{\text{Mean length of longest root in treatment}}{\text{Mean length of longest root in control}} \]

Phytotoxicity

The percentage of phytotoxicity of effluent was calculated by using the formula proposed by (Chou et al., 1978).

\[ \text{Percentage of phytotoxicity} = \frac{\text{Radicle length of control} - \text{Radicle length of test}}{\text{Radicle length of control}} \times 100 \]
PHOTOSYNTHETIC PIGMENTS AND BIOCHEMICAL ANALYSES

The photosynthetic pigments such as chlorophyll “a, b” total chlorophyll and carotenoid and the biochemical contents such as sugars, starch, amino acids and protein were estimated in the seventh day old seedlings grown in the laboratory conditions, pot, plate and field experiments.

Chlorophyll (Arnon, 1949)

Five hundred mg of fresh leaf material was grounded with a mortar and pestle with 10 ml of 80 per cent acetone. The homogenate was centrifuged at 800 rpm for 15 min. The supernatant was saved. The residue was re-extracted with 10 ml of 80 per cent acetone. The supernatant was saved and the absorbance values were read at 645 and 663 nm in a UV-Spectrophotometer (Hitachi). The chlorophyll ‘a’, chlorophyll ‘b’ and total chlorophyll contents were estimated and expressed in mg/g fresh weight basis.

\[
\text{Chlorophyll 'a'} = (0.0127) \times (\text{O.D 663}) - (0.00269) \times (\text{O.D 645})
\]

\[
\text{Chlorophyll 'b'} = (0.0229) \times (\text{O.D 645}) - (0.00488) \times (\text{O.D 663})
\]

\[
\text{Total chlorophyll} = (0.0202) \times (\text{O.D 645}) + (0.00802) \times (\text{O.D 663})
\]

Carotenoid (Kirk and Allen, 1965)

The same extract used for chlorophyll estimation was used for carotenoid estimation. The acetone extract was read at 480 nm in a UV-Spectrophotometer. The carotenoid content was calculated by using the following formula and it was expressed in mg/g fresh weight.

\[
\text{Carotenoid} = (\text{O.D 480}) - (0.114) \times (\text{O.D 663}) - (0.638) \times (\text{O.D 645})
\]
Estimation of protein (Lowry et al., 1951)

Extraction

Five hundred mg of plant materials were weighed and macerated in a pestle and mortar with 10 ml of 20 per cent trichloroacetic acid. The homogenate was centrifuged for 15 min at 600 rpm. The supernatant was discarded. To the pellet, 5 ml of 0.1 N NaOH was added and centrifuged for 5 min. The supernatant was saved and made up to 10 ml with 0.1 N NaOH. This extract was used for the estimation of protein.

Estimation

One ml of the extract was taken in a 10 ml test tube and 5 ml of reagent ‘C’ was added. The solution was mixed and kept in darkness for 10 min. Later, 0.5 ml of folin-phenol reagent was added and the mixture was kept in dark for 30 min. The sample was read at 660 nm in the UV-Spectrophotometer. The protein contents were expressed in mg/g fresh weight.

Preparation of reagents

Reagent A: 0.4 g of sodium hydroxide was dissolved in 100 ml of distilled water. To this solution, 2 g of sodium carbonate was added.

Reagent B: 1 per cent of copper sulphate was mixed with equal volume of 2 per cent sodium potassium tartarate.

Reagent C: 50 ml of reagent A and 1 ml of reagent B were taken and mixed and it was prepared freshly at the time of experiment.

Folin-phenol reagent: 1 ml of folin-phenol reagent was diluted with 2 ml of distilled water.
Estimation of amino acids (Moore and Stein, 1948)

Extraction

Five hundred mg of plant materials were weighed and macerated with a pestle and mortar with 10 ml of 80 per cent ethanol. The homogenate was centrifuged for 10 min at 800 rpm. The supernatant was saved. The extract was used for the estimation of amino acids.

Estimation

One ml of the extract was pipetted out into a test tube. A drop of methyl red indicator was added. The sample was neutralized with 1 ml of 0.1 N sodium hydroxide. To this, 1 ml of ninhydrin reagent was added and mixed thoroughly. The content of the test tube was heated for 20 min in a boiling water bath. 5 ml of the diluent solution was added and heated in water bath for 10 min. The tubes were cooled under the running water and the contents were mixed thoroughly. Blank was prepared without extract. The absorbance was read at 570 nm in a UV-Spectrophotometer. The amino acid contents are expressed in mg/g fresh weight.

Ninhydrin reagent

Eight hundred grams of hydrated stannous chloride was dissolved in 500 ml of citrate buffer at pH 5.0 and 20 g of recrystallized ninhydrin was dissolved in 500 ml of methyl cellosolve. Then these two solutions were mixed.

Estimation of sugars (Nelson, 1944)

Extraction

Five hundred mg of plant materials was weighed and macerated in a pestle and mortar with 10 ml of 80 per cent ethanol. The homogenate was centrifuged for 10 min at 800 rpm. The supernatant was saved. Then, the ethanol is evaporated in a
water bath at 50°C. The net content was made up to 20 ml with distilled water and the extract was used for the estimation of reducing sugar.

**Estimation**

One ml of extract was taken in a 25 ml marked test tube. 1 ml of reagent ‘C’ was added. Then, the mixture was heated for 20 min at 100°C in a boiling water bath, cooled and 1 ml of arsenomolybdate reagent was added. The solution was thoroughly mixed and diluted to 25 ml with distilled water. The sample was read in a UV-Spectrophotometer at 520 nm. The sugar contents were expressed in mg/g fresh weight.

**Preparation of reagents**

**Reagent A:** Twenty five grams of anhydrous sodium carbonate, 25 g of sodium potassium tartarate, 20 g of sodium bicarbonate and 200 g of anhydrous sodium sulphate were dissolved in 800 ml of distilled water and made up to 1000 ml. Then, it was filtered and stored in a glass stoppered brown bottle.

**Reagent B:** Fifteen per cent (per cent) copper sulphate containing 1 or 2 drops of concentrated sulphuric acid.

**Reagent C:** Fifty ml of reagent A and 1 ml of reagent B were mixed well and it was prepared freshly at the time of experiment.

**Arsenomolybdate reagent:**

To 450 ml of distilled water, 25 g of ammonium molybdate, 21 ml of concentrated sulphuric acid were added and 3 g of sodium arsenate was dissolved in 25 ml of distilled water. The mixture was kept in a water bath at 37°C for 24 to 48 hours. The reagent was stored in a glass stoppered brown bottle.
Non-reducing sugars (Nelson, 1944)

Non-reducing sugars present in the ethanol extracts (extraction as in reducing sugars) were hydrolyzed with sulphuric acid to reducing sugars. Reducing sugars present in the hydrolysates were estimated following Nelson’s method. The difference between the total sugars and the reducing sugars correspond to the non-reducing sugars.

Hydrolysis

One ml of extract was taken in a test tube and evaporated to dryness in a water bath for 15 minutes. To the residue, 1 ml of distilled water and 1 ml of 0.1 N sulphuric acid were added. The mixture was hydrolyzed by incubating at 49°C for 30 min in a thermostat. The solution was neutralized with 0.1 N NaOH (5 ml) and the methyl red as indicator. To this, 1 ml of reagent C (copper reagent) was added and heated for 20 min, cooled and 1 ml of arsenomolybdate reagent was added. The content was made up to 25 ml and the absorbance was read at 495 nm in a UV-Spectrophotometer. The reducing sugar contents were expressed in mg/g fresh weight. Blank was prepared with 1 ml of distilled water.

Extraction and estimation of starch (Dubois et al., 1956)

Five hundred mg of plant material was weighed and macerated in a pestle and mortar with 10 ml of 80 per cent ethanol. The sample was centrifuged at 6000 rpm for 15 minutes. The supernatant was removed and the pellets were extracted with 52 per cent perchloric acid for 30 min at 0°C. The extract was centrifuged and supernatant was diluted up to 15 times. One ml of diluted sample was mixed with 2 ml of cold anthrone reagent in ice bath and it was boiled for 10 minutes at 100°C in
a water bath. The content was cooled and the absorbance was read at 630 nm in a UV-Spectrophotometer. The starch was calculated by multiplying with 0.9 to the values obtained from standard curve. The starch contents were expressed in mg/g fresh weight.

MINERALS

**Total nitrogen (Jackson, 1958 quoted by Yoshida et al., 1972)**

Two hundred mg of dried powered plant sample was taken in a 100 ml Kjeldahl flask. Two hundred mg of salt mixture (potassium sulphate, cupric sulphate and selenium powder mixed in the ratio of 50:10:1) and 3 ml of concentrated sulphuric acid was added. After digestion, 10 ml of distilled water was added and cooled.

The diluted sample was decanted into the micro-Kjeldahl distillation flask. To that, 10 ml of 40 per cent sodium hydroxide was added and distilled. The distillate was collected in a conical flask containing 10 ml of 4 per cent boric acid and 3 drops of mixed indicator (0.3 g bromocresol green and 0.2 g methyl red in 400 ml of 90 per cent ethanol). This solution was titrated against 0.05 N HCl. Nitrogen content was estimated using the following formula:

\[
\text{Nitrogen (per cent)} = \frac{(\text{Sample titrate} - \text{blank litre}) \times \text{N of HCl} \times 14 \times 100}{\text{Sample weight} \times 1000}
\]

**Phosphorus (Black, 1965 quoted by Yoshida et al., 1972)**

One gram of dried powdered plant material was digested with 10 ml of acid mixture (nitric acid 750 ml, sulphuric acid 150 ml and perchloric acid 300 ml). The digest was cooled and made up to 50 ml and filtered. One ml of the digest was
mixed with 2 ml of 2 N nitric acid and diluted to 8 ml. One ml of molybdovanadate reagent (25 g of ammonium molybdate in 500 ml of water, 1.25 g of ammonium vanadate in 500 ml of 1 N nitric acid, both were mixed in equal volume) was added, shaken and the absorbance was measured at 420 nm in UV-Spectrophotometer after 20 min. Calibration curve was prepared using potassium dihydrogen phosphate as standard.

**Potassium (Williams and Twine, 1960)**

Dried powdered plant material (0.5 g) was digested in 100 ml Kjeldahl flask using 10 ml of concentrated nitric acid, 0.5 ml of 60 per cent perchloric acid and 0.5 ml of sulphuric acid. The inorganic residue was cooled and diluted with 15 ml of distilled water and filtered through Whatmann No. 42 filter paper. The filtrate was made up to 50 ml with distilled water. The filtrate was used for potassium estimation by Flame photometer and standards were prepared with potassium chloride.

**SOIL ANALYSES**

The soil samples were collected from each plot before sowing and after harvesting and labelled separately. Their physico-chemical properties such as pH, electrical conductivity, nitrogen, phosphorus, potassium, copper, iron and zinc were estimated and recorded.

**pH**

Twenty grams of soil sample was air-dried and 50 ml of distilled water was added and mixed well. The solutions were taken in beaker and the pH of the soil sample was recorded by using a pH meter.
Electrical conductivity

Twenty grams of dried soil sample was taken and dissolved in 50 ml of distilled water and mixed well. The solution was used to measure the conductivity with the help of an electrical conductivity meter.

Available nitrogen (Subbiah and Asija, 1976)

Twenty grams of the soil sample was taken in a flask and 20 ml of distilled water, 100 ml of freshly prepared 0.32 per cent potassium permanganate solution and 100 ml of 2.5 per cent sodium hydroxide were added. The flask was heated and 30 ml of distillate was collected in 50 ml of N/50 sulphuric acid. Excess acid was titrated against N/50 Na OH solution using methyl red indicator. The amount of available nitrogen per cent in the soil was calculated by using the following formula:

Available nitrogen (mg kg\(^{-1}\)) =

\[
\frac{\text{Volume of } 0.02 \text{ N H}_2\text{SO}_4 - \text{Volume of } 0.02 \text{ N NaOH consumed} \times 0.28}{\text{Weight of soil in gram}} \times 1000
\]

Available phosphorus (Jackson, 1958)

One gram of the soil was suspended in 200 ml of 0.002 N sulphuric acid, shaken well and then filtered through Whatmann No. 42 filter paper. To 10 ml of filtrate, three drops of 0.02 per cent 2,4-dinitrophenol indicator was added. Whenever, the solution became yellow, 2 N sulphuric acid was added until the disappearance of the yellow colour. If the solution was colour less after adding the indicator, 4 N sodium carbonate was added till it became colour less. To that
solution, 2 ml of sulphomolybdic acid (ammonium molybdate 25 g in 200 ml; 275 ml con. H₂SO₄ diluted to 700 ml both were cooled, mixed and made up to 1000 ml) and 0.5 ml of chlorostannous acid (25 g SnCl₂.2H₂O in 50 ml of concentrated HCl diluted to 500 ml with water and made up to 1 L with 1.2 N HCl) were added and made up to 50 ml. The solution was shaken well and read in a UV-Spectrophotometer at 660 nm after 5 minutes. Standard graph was prepared using potassium dihydrogen phosphate.

**Available potassium (Jackson, 1958)**

Ten grams of soil was taken in 250 ml conical flask and 100 ml of 1 N ammonium acetate was added to it. The flask was stoppered, shaken intermittently for 10 min and filtered by suction. Ammonium acetate was poured to the soil to get a volume of 250 ml and then evaporated to dryness. Dried samples were ashed in a muffle furnace at 700-800°C for 20-30 minutes. To the residue, 50 ml of 0.1 N HCl was added and warmed gently and the extract was fed to Flame photometer. Potassium chloride was used to prepare the standard solution.

**Available calcium (Yoshida et al., 1972)**

Five grams of soil was extracted with 50 ml of 1 N ammonium acetate. Two ml of the extract was mixed with 2 ml of 5 per cent lanthanum oxide solution and diluted with 10 ml of 1 N HCl. The solution was fed into an Atomic Absorption Spectrophotometer at 211.9 nm. Standard solution was prepared using calcium chloride.
Available magnesium (Jackson, 1958)

Ten grams of soil sample was extracted with 50 ml of 1 N ammonium acetate and the extract was filtered and used for the determination of magnesium. The determination procedure was adopted as in the case of calcium. The amount of magnesium was estimated by using Atomic Absorption Spectrophotometer. Magnesium chloride was used for the standard preparation.

Copper, iron and zinc (Piper, 1966)

Fifty grams of soil was extracted with 100 ml of extraction solution (diethylenetriaminepentaacetic acid – DPTA) and shaken thoroughly for 2 hours. The solution was filtered through Whatmann No. 42 filter paper. The filtrate was read at 568 nm for iron, 324.6 nm for copper and 214 nm for zinc by using the appropriate hollow cathode lamps in Atomic Absorption Spectrophotometer.

Irrigation schedule (Plot)

The experimental plots were irrigated with tap water as control. The quality of tap water did not vary during the experimental periods as per the standards prescribed by Indian Standard Institution for irrigation water.

First irrigation was done to all plots with well water two days before sowing. The different concentrations (10, 25, 50, and 100 per cent) of sugar mill effluent were prepared with tap water. Equal volumes of the tap water as well as diluted effluent were irrigated at an interval of 7 days up to harvest stage.
Morphological parameters

Plant samples were collected randomly at various stages (30, 60, 90 and 120 DAS) of its growth and used for recording morphometric observation like shoot, root lengths, total leaf area, fresh, dry weights and yield of crop plants. Three or Five plants were selected from each concentration including control for recording the various morphological parameters.

Shoot length and root length

Three or five plant samples were collected at 30, 60, 90 and 120 DAS. Their heights were measured by using cm scale and recorded. In another experiment shoot length and root length were measured and recorded.

Total leaf area

The leaf area was calculated by measuring the length and width of the leaf as described by Yoshida et al. (1972).

\[
\text{Leaf area (cm}^2) = K \times \text{length} \times \text{breadth}
\]

Where,

\[K = \text{Kemp’s constant (for dicot leaves 0.66).}\]

Dry matter production

The plant samples taken for morphometric studies were used for determination of fresh weight and dry weight. They were dried in a hot air oven at 80°C for 24 hours and their dry weights were determined by using electrical single pan balance.
Yield and yield components

Three or five plants were used for recording the various yield parameters like number of pods per plant, number of seeds per plant, 100 seeds weight and total yield.

BIOCHEMICAL ANALYSES

Besides morphological parameters, some biochemical constituents such as chlorophyll ‘a’, chlorophyll ‘b’, total chlorophyll, carotenoid, sugars, starch, protein and amino acids were analyzed and recorded at 30, 60, 90 and 120 DAS. The methods of extraction and estimation were mentioned earlier in germination studies.

MINERAL CONTENTS

Both macronutrients (N, P, K, Ca and Mg) and micronutrients (Zn, Cu and Fe) were analyzed and recorded at 30, 60, 90 and 120 DAS). They were estimated by following the methods mentioned earlier in germination studies.

SOIL MICROBIAL POPULATION

The rhizosphere sample of groundnut crop was collected at 30, 60, 90 and 120 DAS after the different concentration of sugar mill effluent irrigation.

Ten gram of soil sample was collected by digging out a block of soil with the plant intact. The soil around the root system was carefully removed. The root portion was gently immersed in 100 ml sterile distilled water in Erlenmeyer flask
and mixed thoroughly by shaking the flask for 5 minutes. Serial dilutions of the suspension were made using sterile distilled water.

One ml sample from these diluted suspensions was removed and transferred aseptically to petridishes and the melted agar medium added. The suspension was mixed well with agar and then allowed to settle. The dishes were incubated at room temperature (28-30°C) for various lengths of time before they were taken up for counting the microbial colonies.

**Bacteria**

The bacterial population in the rhizosphere soil was estimated by employing soil extract agar medium. The colonies were counted on the 3\textsuperscript{rd} day of incubation.

**Fungi**

The fungi in the rhizosphere soil were estimated by employing Martin’s Rose Bengal agar medium. The colonies were counted on the 5\textsuperscript{th} day of incubation.

**Actinomycetes**

The actinomycetes population in the rhizosphere soil was estimated by employing soil extract agar medium. The colonies were counted on the 11\textsuperscript{th} day of incubation.
Media used

Soil extract agar (Allen, 1953)

Glucose - 1.0 g, di-potassium hydrogen phosphate - 0.5 g, agar - 15.0 g, soil extract - 100 ml, tap water - 900 ml and pH - 7.0 - 7.2.

Soil extract was prepared by treating 1000 gram of soil in 1000 ml of tap water and autoclaving for 60 minutes. A small quantity of calcium carbonate was added and the soil suspension was filtered till the extract was clear.

Martin’s Rose bengal agar (Allen, 1953)

Dextrose - 10 gram, peptone - 5 gram, potassium dihydrogen phosphate - 1 gram, magnesium sulphate - 0.5 gram, rose bengal - 1 part in 30,000 parts of medium, agar - 20 gram, distilled water - 1000 ml and pH - 6.5.

One gram of streptomycin sulphate was added in 100 ml sterile distilled water after opening the vial aseptically. 0.3 ml of streptomycin sulphate solution was added to each 100 ml of rose bengal medium.

Base medium-77 (Allen, 1953)

Mannitol - 10 gram, CaCO₃ - 3 gram, K₂HPO₄ - 0.5 gram, MgSO₄.7H₂O - 0.2 gram, sodium chloride - 0.2 gram, manganese sulphate - trace, ferric chloride - trace, agar - 15 gram, double distilled water - 1000 ml and pH - 7.0.
N.F.B. medium (Day and Dobereiner, 1976)

Malic acid - 5 gram, KOH - 4 gram, K$_2$HPO$_4$ - 0.5 g, FeSO$_4$7H$_2$O -48 mg,
MnSO$_4$H$_2$O - 8 mg, MgSO$_4$7H$_2$O - 100 mg, NaCl - 16 mg, CaCl$_2$ - 8 mg,
Na$_2$MOO$_4$ - 0.008 mg, agar - 15 g, pH 6.6 - 7.0 and distilled water - 1000 ml.

STATISTICAL ANALYSIS

The statistical analysis of experimental results was carried out by standard deviation. In order to analyze the data statistical tool such as ANOVA (Analysis of Variance) was used. Standard deviation calculated by following methods of O’Brien, R. G. (1981); Neter, J., Wasserman, W., and Kutner, M. H. (1990).