III. MATERIALS AND METHODS

The present investigation was carried to find out the effect of different concentrations of tannery effluent on germination, growth and yield response of horse gram (*Dolichos biflorus* L.) varieties. The present research work comprises the following aspects: (i) screening of the horse gram varieties for tolerance to tannery effluent (ii) changes in biochemical and mineral contents of tolerant variety of horse gram and (iii) suitable inoculants bio-fertilizers under tannery effluent.

3.1. Materials

3.1.1. Effluent sample

The effluent sample was collected in plastic containers directly collected from the outlet of effluent at K.R. leathers, Ranipet, at Vellore District, Tamil Nadu. It was brought to for their physico-chemical analyses. The effluent sample was stored in refrigerator to avoid any microbial decomposition.

The effluent sample was analysed for their physico-chemical properties in the private laboratory, following the routine standard methods mentioned in American Public Health Association (APHA, 1998).

Materials

Seed materials

Seven varieties of horse gram (*Dolichos biflorus* L.) varieties Paiyur 1, Paiyur 2, CO 1, CO 2, BJPL 1, BJPL 2 and DPI 2278 were obtained from Paiyur Research Stations, of Tamil Nadu Agricultural University (TNAU) located at Dharmapuri District, Tamil Nadu, India. The healthy seeds were chosen and used for both laboratory and field experiments.
Bio-fertilizers

The collection of bio fertilizers like *Rhizobium*, *Azospirillium*, and Phosphobactria were procured from Tamil Nadu Agriculture University TNAU Coimbatore.

Methods

**Physico-chemical analyses of tannery 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.

**Temperature (°C)**

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

**pH**

The pH of the effluent sample was measured by a pH meter using a glass electrode as soon as it was collected.

**Electrical conductivity**

The specific conductance of the effluent sample was measured by using a self-contained conductivity meter at 25 °C. It is expressed as μmhos/cm.
**Total solids**

Total solids were determined by taking 100 ml of sample in a pre-weighed evaporating disc and the sample was evaporated to dryness at 103 °C for an hour. The amount of solids is expressed in mg/l.

**Suspended solids**

Suspended solids were estimated by passing through Whatmann No. 4 filter paper and the residue obtained was dried to constant weight at 104 – 105 °C. It is only the difference between total solids and dissolved solids.

**Dissolved solids**

The dissolved solids were estimated by filtering 100 ml of well mixed samples through a standard glass fiber filter. The filtrate was evaporated to get constant weight at 103 °C.

**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. One ml 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.

**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 by 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 of dissolved oxygen.

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

**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 hrs in a water bath. After cooling, the solution was made upto 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.

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

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

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

**Calcium**

Fifty ml of effluent sample was taken in a conical flask. Five ml of triethanolamine and a 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.

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

**Sulphate**

Hundred ml of sample was taken in a conical flask. Ten ml of Con. 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 filtered the precipitate through Whatmann No. 42. filter paper. It was dried, cooled, weighed and calculated the sulphate present in the given sample.

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

**Nitrate**

A known volume of sample was taken in a porcelain basin and evaporated to dryness. 0.5 ml of phenol disulphonic acid was added to the residue. It was dissolved with the help of a glass spatula. 5 ml of distilled water and 1.5 ml of potassium hydroxide solution were added. The supernatant of yellow colour was taken and its absorbance was read in UV-Spectrophotometer. A blank was also run in similar manner with distilled water. The standard titrate solutions were processed in similar manner for each absorbance and concentrations of various standard solutions were drawn. The volume of nitrate and nitrogen in the sample were reduced by comparing the absorbance of sample with the standard curve.
Per cent sodium

Digested sample of the effluent was subjected to determine 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 the potassium content.

Sodium can be determined by dividing the percentage of the sodium concentration to the cationic concentration.

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

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.

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

Preparation of different concentrations of tannery effluent

The collected effluent sample from the outlet of tannery effluent was considered as 100 per cent raw effluent. Different concentrations (10, 20, 30, 40, 50 and 100 per cent) of tannery effluent were prepared freshly by using tap water whenever necessary. They were used for germination studies.

Control : Tap water

10% : 10 ml effluent + 90 ml of water (for 100 ml)

20% : 20 ml effluent + 80 ml of water
30% : 30 ml effluent + 70 ml of water
40% : 40 ml effluent + 60 ml of water
50% : 50 ml effluent + 50 ml of water
100% : Raw effluent (undiluted)

Germination study-I: Varietal screening experiment

The healthy seeds of horse gram varieties (Paiyur 1, Paiyur 2, CO 1, CO 2, BJPL 1, BJPL 2 and DPI 2278) were surface sterilized with 0.1 per cent mercuric chloride for 2 minutes and washed thoroughly with tap water and then with distilled water. Twenty seeds of seven varieties of horse gram were arranged equispacially in plastic trays lined with filter paper. They were irrigated uniformly with equal volume of different concentrations (10, 20, 30, 40, 50 and 100 per cent) of tannery effluent. The seeds irrigated with tap water were treated as control. They were allowed to grow for 10th days. Three replications were maintained for this varietal screening experiment. On the basis of the data obtained from varietal screening experiments, the variety Paiyur -2 was identified as tolerant variety when compared with other varieties tested. So, the tolerant variety Paiyur -2 was chosen for further experiments.

Germination per centage

The number of seeds germinated in each treatment was counted on each and every day upto 10th day after sowing. The seed germination percentage was calculated by using the following formula:

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 10th day from each treatment to record the seedling growth. The growth of the seven varieties of horse gram seedlings was measured by using a centimeter scale and the values were recorded.

Seedling dry weight (g/seedling)

Twenty seedlings were kept in a hot air oven at 75 ºC for 24 hrs. Then, the samples were kept in a desiccator and their dry weights were taken by using an electrical single pan balance. The average is expressed in g/seedling.

Vigour index

Vigour index of the seedlings was 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 was calculated by using the formula proposed by Turner and Marshal (1972).

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

Percentage of phytotoxicity

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

Photosynthetic pigments and biochemical analyses

The photosynthetic pigments such as chlorophyll and carotenoid and the biochemical contents such as sugars, starch, amino acids and protein were estimated in the 10th day old seedlings grown in the laboratory conditions.

Chlorophyll (Arnon, 1949)

Five hundred mg of fresh leaf material was ground 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.

\[
\begin{align*}
\text{Chlorophyll ‘a’} & \quad = \quad (0.0127) \times (\text{O.D. 663}) - (0.00269) \times (\text{O.D. 645}) \\
\text{Chlorophyll ‘b’} & \quad = \quad (0.0229) \times (\text{O.D. 645}) - (0.00488) \times (\text{O.D. 663}) \\
\text{Total chlorophyll} & \quad = \quad (0.0202) \times (\text{O.D. 645}) + (0.00802) \times (\text{O.D. 663})
\end{align*}
\]

Carotenoid (Kirk and Allen, 1965)

The extract used for chlorophyll estimation was also used for carotenoid estimation. The acetone extract was read at 480 nm in a UV-Spectrophotometer (Hitachi). The carotenoid content was calculated by using the following formula and it is expressed in mg/g fresh weight.
Carotenoid = (O.D 480) – (0.114) × (O.D 663) – (0.638) × (O.D 645)

**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. Five 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**

800 g 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 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 trichloro acetic 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 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:** One per cent of copper sulphate was mixed with equal volume of 2 per cent sodium potassium tartarate.

**Reagent C:** Fifty 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:** One ml of folin-phenol reagent was diluted with 2 ml of distilled water.

**Estimation of total sugar (Nelson, 1944)**

**Extraction**

Five hundred mg of plant materials were 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 was evaporated
in a water bath at 50 °C. The net content was made upto 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 are 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 upto 1000 ml. Then, it was filtered and stored in a glass stoppered brown bottle.

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

**Reagent C:** Fifty ml of reagent A and one 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 hrs. The reagent was stored in a glass stoppered brown bottle.
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% ethanol. The sample was centrifuged at 6000 rpm for 15 min. The supernatant was removed and the pellets were extracted with 52% 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 min 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 are expressed in mg/g fresh weight.

Mineral contents

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

Two hundred mg of dried powered plant sample was taken in a 100 ml Kjeldhal 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 bromocerol 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,
\[
\text{Nitrogen } (\%) = \frac{(\text{Sample titrate - blank litre})N \times 14 \times 100}{\text{Sample weight} \times 1000}
\]

**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 upto 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 of 0.5 g was digested in 100 ml Kjeldahl flask using 10 ml of concentrated nitric acid, 0.5 ml of 60% 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 upto 50 ml with distilled water. The filtrate was used for potassium estimation by Flame photometer and standards were prepared with potassium chloride.

**Calcium and magnesium (Yoshida et al., 1972)**

Two ml of the filtrate was mixed with 2 ml of 5 per cent lanthanum chloride solution and diluted with 10 ml of 1 N hydrochloric acid. The solution was fed into an Atomic Absorption Spectrophotometer at 211.9 nm for calcium and 285.4 nm for
magnesium. Standard curve was prepared by using calcium chloride/magnesium chloride.

**Pot culture (II Experiment)**

Pot culture experiment was conducted to find out the suitable tolerant variety (Paiyur - 2) application of bio fertilizers in soil the and tannery effluent irrigation. The growth and yield parameters (root length, shoot length, total leaf area, total dry weight and yield) were recorded at 30, 60 and 90 DAS. On the basis of the morphological growth and yield parameters, the tolerant variety (Paiyur 2) was selected.

**Irrigation schedule**

The experimental pots were irrigated with well water was treated 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 pots with well water two days before sowing. Since no germination was recorded in 10, 20, 30, 40, 50 and 100 per cent concentration tannery effluent was prepared and used for this pot culture experiment. Equal volumes of the tap water as well as effluent were irrigated at an interval of 10 days up to harvest stage.

**Morphological parameters**

Plant samples were collected randomly at various stages (30, 60 and 90 DAS) of its growth and used for recording morphometrical observations like root length, shoot length, total leaf area, total dry weight and yield of crop plants. Five plants were selected from each treatment including control for recording the various morphological parameters.
Root length and Shoot length

Five plant samples were collected from each treatment plot at every 30, 60 and 90 DAS. Their heights (shoot length and root length) were measured by using centimeter scale and expressed in cm/plant.

Total leaf area

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

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

Where

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

Total dry weight

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

Yield and yield components

Five plants were used for recording the various yield parameters like number of pods per plant, dry weight of pods, and total yield.

Field experiment-II (Tolerant variety)

Experimental period : April 2010 to July 2010.

Variety : Paiyur- 2

Experimental design : Randomized black design

Treatments : 10, 25, 50, 75 and 100 per cent tannery effluent and bio fertilizers (Rhizobium, Azospirillum and Phosphobacteria)
Replicates : Five
Sampling days : 30, 60 and 90 DAS
Parameters studied : Root length, shoot length, total leaf area, total dry weight and yield parameters

Soil analysis

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, moisture content, organic carbon, available nitrogen, available phosphorus, available potassium, available calcium, available magnesium, available copper, available 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 (μmhos/cm)

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.

Moisture content (%)

Hundred grams of soil samples were collected and oven dried. The oven dried samples were weighed and the percentage of moisture content was calculated by using the formula.

\[
\text{Moisture content (\%)} = \frac{\text{Weight loss after drying}}{\text{Soil dry weight}} \times 100
\]
**Organic carbon**

Soil sample (0.5 g) was taken in a conical flask. Ten ml of 1N K$_2$Cr$_2$O$_7$ solution was added and stirred for a while and then 20 ml of concentrated H$_2$SO$_4$ was added. It was again stirred gently for 40 minutes. The volume was made upto 100 ml with distilled water and mixed thoroughly. The solution was centrifuged and read at 660 nm. The amount of organic carbon present in the sample was determined by comparing the calibration curve.

**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 NaOH solution using methyl red indicator. The amount of available nitrogen in the soil was calculated by using the following formula:

\[
\text{Available nitrogen (mg kg}^{-1}\text{)} = \frac{\text{Volume of 0.02 N H$_2$SO$_4$} - \text{Volume of 0.02 N NaOH consumed} \times 0.28 \times 1000}{\text{Weight of soil in gram}}
\]

**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 colourless after adding the indicator, 4 N sodium carbonate was added till it became colourless. To that
solution, 2 ml of sulphemolybdic acid (ammonium molybdate 25 g in 200 ml; 275 ml con. H₂SO₄ diluted to 700 ml both were cooled, mixed and made upto 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 upto one litre with 1.2 N HCl) were added and made upto 50 ml. The solution was shaken well and read in a UV-Spectrophotometer at 660 nm after 5 min. Standard graph was prepared using potassium dihydrogen phosphate.

**Available potassium (Jackson, 1958)**

Ten grams of soil sample 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 min. 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 sample 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 by 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.

**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, et al., (1990).