CHAPTER II

EXPERIMENTAL AND ANALYTICAL PROCEDURES
2.1 ESTIMATION OF TOTAL CARBON

The total carbon in a sample of soil or other carbonaceous materials, such as sunnhemp and leuconine, was estimated by the method of Robinson, McLean and Williams (1929).

Five grams of well-dried and powdered soil were taken in a Kjeldahl flask. To this, a few crystals of copper sulphate followed by 5 g of fused potassium sulphate were added. The Kjeldahl flask containing these substances was connected with three conical flasks. Standard iodine solution was put in the first conical flask, the second conical flask contained a few ml of iodine solution, while pure potassium iodide solution was kept in the third conical flask. A current of purified air, obtained by passing air through a solution of ferrous sulphate and concentrated sulphuric acid was now aspirated first through the Kjeldahl flask and then through the solutions of iodine. The Kjeldahl flask was then heated directly on a bunsen flame for 4-5 hours. The sulphur dioxide produced by the reaction:

\[ C + 2H_2SO_4 = CO_2 + 2SO_2 + 2H_2O \]  (i)

was carried along with the air current into the iodine solution in the first flask. The sulphur dioxide left unreacted in the first flask was used up in the iodine solution contained in the second flask whilst any iodine
vapours carried along by the current of air bubbling through the iodine solutions were trapped in the third conical flask containing potassium iodide solution. The reaction in the iodine trap is represented as follows:

\[ \text{SO}_2 + \text{I}_2 + 2\text{H}_2\text{O} = \text{H}_2\text{SO}_4 + 2\text{HI} \quad (\text{ii}) \]

The excess of iodine left over after reaction (ii) was titrated against a standard sodium thiosulphate solution and from the amount of iodine used, the value of total carbon was calculated. It follows from equation (i) and (ii) that

One atom of carbon = 2SO₂ = 2I₂

or 12 grams by weight of carbon = 508 grams by weight of iodine

This method is believed to record a little less carbon than is actually present, but, as the initial and the subsequent readings are all taken by the same method, the results obtained are comparative and valid.

2.2 ESTIMATION OF TOTAL NITROGEN

The total nitrogen present in the soil samples was estimated by salicylic acid reduction method (Treadwell and Hall, 1947).

Five grams of the well dried and powdered soil were taken in a 500 ml Kjeldahl flask. To this 30 ml of strong
sulphuric acid, in which one gram of salicylic acid was dissolved, was added. The acid was allowed to react with the soil in the cold for 30 minutes with occasional shaking of the Kjeldahl flask so that the nitrate may combine with salicylic acid according to the reaction,

\[ 2\text{KNO}_3 + \text{H}_2\text{SO}_4 \rightarrow \text{K}_2\text{SO}_4 + 2\text{HNO}_3 \]

\[ \text{HNO}_3 + \text{HO-}\text{C}_6\text{H}_4\text{COOH} \rightarrow \text{HO-}\text{C}_6\text{H}_3\text{NO}_2\cdot\text{COOH} + \text{H}_2\text{O} \]

After 30 minutes of this treatment 5 grams of sodium thiosulphate were added which reduced the nitrate group to form amino-salicylic acid as shown below:

\[ \text{Na}_2\text{S}_2\text{O}_3 + \text{H}_2\text{SO}_4 \rightarrow \text{Na}_2\text{SO}_4 + \text{H}_2\text{SO}_3 + \text{S} \]

\[ 3\text{H}_2\text{SO}_3 + \text{HO-}\text{C}_6\text{H}_3\text{NO}_2 \rightarrow \text{COOH} + \text{H}_2\text{O} \rightarrow 3\text{H}_2\text{SO}_4 + \text{HO-}\text{C}_6\text{H}_3\text{NH}_2\cdot\text{COOH} \]

The mixture was now heated, when all the carbon obtained by charring had disappeared and the froth had stopped. Eight grams of potassium sulphate and a few crystals of copper sulphate were then taken and added to the Kjeldahl flask, which was regularly shaken to ensure complete reduction of nitrate. The digestion was completed by heating it for another four hours in a fuming chamber. After cooling, the mixture was diluted with 100 ml of distilled water and boiled for a few minutes, in order to break down any cement.
like material formed during this process. This solution containing total nitrogen as ammonium sulphate was filtered, washed and made up to a definite volume. An aliquot of this solution was now distilled with 100 ml of 40% sodium hydroxide solution. The issuing gas (as shown by the following reaction), was received in 20 ml of N/10 sulphuric acid contained in a conical flask.

\[(\text{NH}_4)_2\text{SO}_4 + 2\text{NaOH} \rightarrow 2\text{NH}_3 + 2\text{H}_2\text{O} + \text{Na}_2\text{SO}_4\]

100 ml of distilled water was added to the acid followed by a few drops of methyl red indicator. The whole of sulphuric acid solution after the reaction was titrated against approximately N/10 (as standardised against N/10 oxalic acid) sodium hydroxide solution, which gave the amount of acid used up and hence the ammonia evolved was calculated.

2.3 TOTAL NITRATE

Ten grams of an oven dried sample (temperature maintained at 55°0 for 16 hours) was taken. The dried soil was transferred to a 9 cm Buchner funnel fitted with a Whatman (No. 50) filter paper, and sufficient distilled water containing 10 drops of concentrated sulphuric acid was poured. After a few minutes this was connected to the filter pump and the leaching of the soil was continued with successive quantities of distilled water till the filtrate
amounted to about 600 ml. The filtrate was then transferred to a one litre Erlenmeyer flask and one gram of magnesium oxide was added. The solution was then evaporated until the volume was reduced to about 200 ml. It was allowed to cool and then 5 g of zinc dust, 70 ml of 30% sodium hydroxide and 5 g of powdered iron were added in the above order. The litre flask was then connected to the condenser of the nitrogen distillation apparatus through an efficient splash trap. The reduction was done in cold for about half an hour and then over a very small flame while the ammonia was boiled off in the third period in approximately half an hour. The ammonia was collected in 20 ml of 0.02 N hydrochloric acid and the excess of the acid was back titrated using 0.02N sodium hydroxide and methyl red as indicator. The amount of nitrate nitrogen in mg per kg (ppm) was calculated with the help of expression:

\[
(B - T) \times N \times 14 \times \frac{1000}{W}
\]

Where,

B = blank titration in ml of standard alkali,
T = actual titration in ml of standard alkali,
N = Normality of standard alkali,
W = Weight of soil taken.
2.4 **TOTAL AMMONIA**

Ten grams of the soil was taken in a 400 ml beaker. 100 ml of a cold 1N solution of sodium chloride was added. It was then thoroughly stirred and left to stand for half an hour. The supernatant liquid was decanted through 18.5 cm Whatman (No. 44) filter paper and the filtrate was collected in a litre Erlenmeyer flask. After washing the soil once by decantation with the normal sodium chloride solution it was transferred completely to the filter paper. The leaching was continued until the volume of the filtrate was about 500 ml.

Three to four grams of magnesia was added to the filtrate, which was then connected to the ammonia distillation apparatus. It was heated gently so that a volume of 150-220 ml distilled over in half to three-quarters of an hour. The distillate was collected in 20 ml of 0.02N hydrochloric acid and the excess of the acid was back titrated with 0.02N sodium hydroxide, using methyl red as indicator. A blank determination was carried out using all the reagents.

The amount of ammonical nitrogen in \( \text{mg} \) ppm corresponds to:

\[
\frac{(B - T) \times \text{Normality factor} \times 14 \times 10000}{\text{weight of soil taken}}
\]
Where,

\[ B = \text{Volume of standard sodium hydroxide used in the blank determination.} \]

\[ T = \text{Volume of standard sodium hydroxide used in the actual determination.} \]

The value was expressed on oven-dry soil basis.

2.5 **ANALYSIS OF MINERAL CONSTITUENTS OF SOIL**

The method employed for the analysis of mineral constituents present in the soil were those described for soil analysis by C.H. Wright (1934) and the method published by the Association of Official Agricultural Chemists (1945). All soil samples were analysed in duplicate to obtain concurrent results.

2.5.1 **Moisture**: The moisture contents were determined by heating 10 g of the air-dried soil in an oven at a temperature of 105-110°C for about four hours. The loss in weight gave the moisture content.

2.5.2 **Organic matter**: 10 g of the oven-dried soil was taken in silica crucibles and was ignited, firstly slowly then strongly for five-six hours till it became reddish brown or greyish brown in colour. The whole mass was thoroughly
mixed for two or three times with a fine glass rod or silver wire to ensure complete ignition of the organic matter. The crucibles were allowed to cool in a desiccator and then weighed. The process was repeated till a constant weight was obtained. The differences in weight after ignition gave the amount of organic matters present in 10 g of the soil.

2.5.3 Preparation of Hydrochloric acid extract and estimation of silica: The HCl acid extract employed for the soil analysis was prepared according to the procedure given by the Agricultural Education Association. The ignited soil from the above experiment was carefully transferred to a 600 ml pyrex glass beaker by means of a glass rod and 100 ml of conc. HCl acid (B.P. 110°C and S.G. 1.10) were added to it. The contents were then heated to boiling on a sand bath for an hour. The contents were allowed to cool and 100 ml of distilled water was added to it. The diluted solution in the beaker was filtered through a Whatman (No. 42) filter paper. The filtrate was collected in a 500 ml graduated flask together with the washings of the residue. This was repeated till the residue was free from the chlorides ions. The insoluble portion left on the filter paper was dried in an air-oven and estimated for silica.

The hydrochloric acid extract was made up to 500 ml and was used for the estimation of sesquioxide, iron oxide,
calcium oxide, magnesium oxide, phosphorus pentoxide and the oxide of potassium according to the following methods:

2.5.4 *Sesquioxides*: 100 ml of the hydrochloric acid extract was taken in a pyrex beaker and 2 g of ammonium chloride was added to it. The sesquioxide was precipitated by the addition of cold dilute ammonium hydroxide (1 + 1). The beaker was now heated to boiling over a sand bath for an hour and the contents were filtered when hot. The precipitate was washed with boiling water till free from chloride ions. The filtrate along with the washings was collected in a flask for subsequent analysis. The precipitate on the filter paper was dried, ignited, cooled and weighed to a constant weight and calculated as under:

\[ \text{Percentage (\%)} \text{ of } \frac{\text{Weight of sesquioxide}}{\text{in the sample}} \times 10 \times 5 \]

2.5.5 *Iron oxide*: 100 ml of the hydrochloric acid was taken and the sesquioxide was precipitated, as given above. The precipitate after being washed completely for chloride ions was transferred to a conical flask of 500 ml. 30 ml of 30% sulphuric acid was added to the flask and mixed. 0.5 g of zinc dust. The flask was kept overnight after corking it with a Bunsen valve to reduce all the ferric ions to the ferrous state. It was filtered with the help
of glass wool and the filtrate was titrated against N/10 KMnO₄ solution. The iron oxide was calculated as follows:

\[
\text{Percentage (\%)} = \text{ml of N/10 KMnO₄ required \times 5 \times 0.008}
\]

iron oxide

2.5.6 Calcium oxide: The filtrate obtained from the sesquioxide precipitation was reduced to 100 ml and was made alkaline by adding a dilute solution of ammonium hydroxide. The solution was boiled and calcium precipitated by adding a slight excess of a warm saturated ammonium oxalate solution. The contents of the beaker were stirred, boiled and kept for 4 hours. The clear solution at the top was decanted on a Whatman (No. 44) filter paper and the precipitate was washed with hot water till free from oxalate ions. The precipitate in the beaker and on the filter paper was now dissolved in dilute hydrochloric acid (about 80%). The calcium was reprecipitated by boiling the solution and by adding a little hydroxide and then ammonium oxalate. The precipitate was allowed to stand overnight and was filtered through a filter paper and the oxalate ions were washed with few aliquots of hot water. The filtrate and the washings were collected in the same beaker containing the first filtrate of the previous precipitation for the estimation of magnesium. The precipitate of calcium oxalate was dissolved in 30 ml of 30% sulphuric acid and the solution after being warmed was titrated, against N/10 KMnO₄. The calcium oxide was calculated as:
Percentage (%) of \( = \text{ml of } \frac{N}{10} \text{KMnO}_4 \text{ required } \times 5 \times 10 \times 0.0028 \) calcium oxide

2.5.7 **Magnesium oxide**: The volume of the filtrate left after the precipitation of calcium was reduced to 50 ml and 5 ml of strong HCl with a drop of methyl orange were added to it. The acid was just neutralised by adding dilute solution of ammonium hydroxide. 10 ml of a saturated solution of sodium dihydrogen phosphate was then added to this solution. The solution was stirred and made alkaline by adding ammonium hydroxide in excess and the precipitate thus obtained was kept for about four hours. It was filtered through a Whatman (No. 44) filter paper and made free of phosphate by washing it with dilute ammonium hydroxide. The precipitate of magnesium–ammonium–phosphate, was dissolved in 10 ml of nitric acid (80%) and was reprecipitated following the above procedure. The precipitate was washed, dried, ignited and finally weighed as \( \text{Mg}_2\text{P}_2\text{O}_7 \). The magnesium oxide in the sample was thus calculated as:

\[
\text{Percentage (\%)} = \text{weight of ppt } \times 5 \times 10 \times 0.36213 \\
\text{magnesium oxide}
\]

2.5.8 **Phosphorus pentoxide**: 50 ml of the hydrochloric acid extract was taken in a beaker and was evaporated to dryness on a sand bath. After cooling 5 ml of conc. nitric acid
was added and was again gently heated and diluted. The
phosphate was precipitated by adding some freshly solution of
prepared ammonium molybdate. The precipitate was left
overnight. It was filtered, washed with first 1% nitric
acid and then finally with 3% of potassium nitrate solution
till it was free of acid. The acid free precipitate was
transferred to the same beaker and 10 ml of standard N/10
sodium hydroxide was added. The excess of sodium hydroxide
left unused was titrated against N/10 nitric acid using
phenolphthalein as indicator. The sodium hydroxide
solution was standardised with the help of a standard oxalic
acid solution. The percentage of phosphorus was thus
calculated as:

\[
\text{Percentage (\%)} \text{ of phosphorus pentoxide} = \frac{\text{ml of N/10 NaOH required}}{10 \times 10 \times 0.000309}
\]

2.5.9 Potassium oxide: 50 ml of hydrochloric acid extract
was evaporated in the same way as discussed earlier in
phosphorus pentoxide determination. 5 ml of glacial acetic
acid was added to it after cooling. The mixture was stirred
and 10 ml of a saturated solution of sodium chloride and
5 ml of sodium nitrite were added. The whole mixture was
then stirred vigorously and 0.5 ml of cobalt nitrate
solution was added to precipitate potassium as potassium
cobaltinitrite. The contents of the beaker were stirred and kept overnight. The precipitate was filtered through a Geech crucible and washed with cobalt nitrate solution. The precipitate together with the crucible was placed in the same beaker containing a known volume of standard (N/10) KMnO₄ to which 30% sulphuric acid was added. The contents of the beaker were heated on sand bath to boiling. While hot, a required quantity of N/10 oxalic acid (N/10) was added. The permangmate colour was discharged. The excess of oxalic acid was back titrated with N/10 KMnO₄ solution. The potassium contents were calculated as:

\[
\text{Percentage (\% of potassium oxide) = ml of N/10 \times 10 \times 10 \times 0.000856} \quad \text{KMnO}_4 \text{ used}
\]

2.6 **MINERAL ANALYSIS OF PLANT MATERIALS**

25 g of the finely powdered plant (complete plants including roots and nodules) of sunnhemp or lucerne was taken in a silica crucible (basin of about 50 ml capacity). It was heated by a low bunsen flame for 6-8 hours until the moisture was removed. Next day the silica basin was placed in an oven at 300°C, where the materials were charred and glowed faintly. This operation was carried till the whole carbon of the plants disappeared. The temperature of the Muffle furnace was then raised to 500°C and the ashing
continued for 8 hours till the colour of the ash became greyish white. The crucible was cooled overnight in a desiccator and weighed. The percentage of the ash was obtained by difference.

The ash contained in the crucible was leached with water and transferred to a beaker carefully using 40 ml of dilute hydrochloric acid. The mixture was heated on a water bath and few drops of concentrated nitric acid were added to oxidise any ferrous salt to ferrie state. The solution was then evaporated to dryness. The heating was further continued for an hour on the water bath and finally in an oven at 105°C to dehydrate the silica. After complete drying, the dried mass was extracted with 100 ml of concentrated hydrochloric acid (50%) and boiled on a water bath. It was then filtered through a filter paper and the filtrate was collected in a 500 ml flask. The residue was washed first with 50% hydrochloric acid and finally with hot water till free from chloride ions. The residue which was mainly silica was dried, ignited as usual and weighed in a crucible to a constant weight.

The hydrochloric acid extract so prepared, was used for the estimations of sesquioxides, iron oxide, calcium, magnesium, phosphorus and potassium constituents of the plant material. The methods employed were exactly the same as described earlier for the analysis of soils.
2.7 MAXIMUM ERRORS IN ESTIMATIONS:

10 g of the material was taken in a silica crucible of 50 ml capacity and was heated on a low Bunsen flame for 6-8 hours to release the moisture. Now the crucible was placed in a Muffle furnace kept at 300°C to char the contents. The heating was continued till the whole carbon disappeared. The temperature of the furnace was then raised to 500°C to convert the materials to ash. The ashing continued for 8 hours till the ash attained a greyish white colour. The crucible was cooled overnight in a desiccator and weighed. The percentage of the ash was determined by difference.

2.7.1 Treatment of the ash for estimation of silica and other mineral constituents:

The ash contained in the crucible was leached with water and transferred to a beaker using 40 ml of dilute hydrochloric acid. The beaker was heated on water bath. Few drops of conc. nitric acid were added to oxidise the ferrous salts present if any, to ferric state. The solution was evaporated to dryness and the heating was continued for an hour on the water bath and finally in an electric oven kept at 105°C for dehydrating the silica. After thorough drying, the dried mass was extracted with 100 ml of conc. hydrochloric acid (50%) and boiled on a water bath.
It was then filtered and the filtrate was collected in a 500 ml flask and was kept overnight in a desiccator. A portion of it was then weighed and used for estimation. All the estimations were carried out in duplicate. In most of the cases an average value of two independent estimations which was very nearly same, was considered. The results have been expressed on oven-dry basis.

Errors in estimation of Carbon and Nitrogen

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<th>% of carbon</th>
<th>% of nitrogen</th>
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</thead>
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<td>1st estimation</td>
<td>0.4043</td>
<td>0.0348</td>
</tr>
<tr>
<td>2nd estimation</td>
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<tr>
<td>3rd estimation</td>
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<td>0.0345</td>
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<tr>
<td>Mean</td>
<td><strong>0.4043</strong></td>
<td><strong>0.0348</strong></td>
</tr>
</tbody>
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The maximum error in the estimation of carbon by Robinson, McLean and William's method is about 0.3% and that of Nitrogen by salicylic acid reduction method is 0.8%.

2.8 **MICROBIAL ANALYSIS** (Azotobacter count):

0.1 g of the air dried soil was carefully weighed and transferred to sterilised boiling test tube containing 10 ml of autoclaved tap water, under aseptic conditions. The
test tube was shaken for about 5 minutes and then allowed
to stand for about half a minute. A series of sterilised
dilution tubes containing 9 ml of autoclaved tap water were
taken and 1 ml of supernatant liquid from the original tube
was transferred by a sterile 1 ml pipette to the first
dilution tube with proper precautions and under aseptic
conditions and the mixture in the first dilution tube was
well shaken. Further dilutions were carried out in the
same way up to the limit which was found by experience,
until 1 ml of the mixture in the last dilution tube, when
plated, allowed the growth of a convenient number of colonies
of the Azotobacter, which could be counted. Platings were
done by transferring 1 ml of the suspension from the final
dilution tube to sterilised petri-dishes and pouring out
about 10 ml of melted and sterilised Beijerinck's medium
(prepared as described below). The petri-dishes were shaken
by giving them a rotatory motion to make the mixture
homogeneous and to spread if evenly on the surface of the
dishes so as to ensure an even distribution of colonies all
over the surface of the petri-dishes. The plates were now
left on a level surface till the medium solidified. The
plates were then incubated at 30°C in an inverted position
for about seven days and the number of colonies of the
Azobacter that developed were counted. The count included
only greyish raised round colonies, semi transparent with
whitish centres and also deep white colonies elliptical or spindly shaped. An average of four plates had been taken.

The following medium was prepared and sterilised at 15 lbs pressure for 20 minutes in an autoclave.

- **Tap water**: 1 litre
- **Mannite**: 20 g
- **Dipotassium phosphate**: 0.2 g
- **Agar**: 20 g

Ten ml portions of the above melted medium were taken in clean boiling test tubes and the tubes plugged with sterile cotton wool and sterilised in the autoclave at 15 lbs pressure for 20 minutes. These tubes were heated in a water bath till the medium melted. The water bath was then allowed to cool down to a temperature of 40°C and one tube was poured into each petri-dish at the time of plating, leaving behind the sediment in the test tube so that a clear medium would set in the plate.

In all cases where Azotobacter counts were made, the moisture contents of the samples taken, were also determined. From the amount of moisture present in a given samples, the number of Azotobacter were calculated on dry basis.
2.9 EXPERIMENTAL DETAILS

200 g of the soil was taken in plastic plates of eight inches diameter. Calculated amounts (corresponding to 0.5% carbon) of finely powdered sunn hemp or lucerne plants were added to every plate. Various phosphates corresponding to their different doses were also added. About 20% moisture in the form of distilled water was introduced in every plate. Two exactly similar sets were prepared, one was exposed to the sunlight, while the other similar set was covered with a thick black paper and placed inside the cupboard to protect it from sunlight. The contents of the plates were stirred on alternate days with a glass rod (using a separate glass rod for each plate) to facilitate aeration and oxidation. The moisture content of each plate was maintained at 20% by adding distilled water, whenever found necessary. After definite intervals (about 30 days) samples from each plate after allowing them to become air dry were analysed. A suitable quantity of the oven dried sample was analysed for its total carbon and total nitrogen content. Azotobacter counts were taken at the intervals of 90 and 180 days using the air dry samples for it.

3.0 CALCULATION OF EFFICIENCY OF NITROGEN FIXATION:

Dhar while explaining photo-synthesis in plants, postulated that the important photochemical reaction is the
decomposition of water by absorption of light according to the following equation:

\[ \text{H}_2\text{O} + 112 \text{ K.cal} = \text{H} + \text{OH} \]

For fixing 14 g of nitrogen and forming ammonia by the interaction of molecular nitrogen and atomic hydrogen obtained by the decomposition of water as stated above, 336 K cal are needed. Hence, from the oxidation of one gram mole of glucose according to the equation:

\[ \text{C}_6\text{H}_{12}\text{O}_6 + 6\text{O}_2 = 6\text{CO}_2 + 6\text{H}_2\text{O} + 676 \text{ K cal.} \]

Under ideal conditions, \( \frac{14 \times 676}{336} \) g of nitrogen can be fixed. In other words, 0.39 g of nitrogen should be fixed per gram of carbon oxidised. Therefore, if 150 mg of nitrogen is fixed per gram of carbon oxidised the efficiency would be \( \frac{150 \times 100}{390} = 38\% \). Similar method for the calculation of efficiency was also used by Dhar and Gupta (1961).
REFERENCES


Wright, C.H. 1934 Soil Analysis.