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Appendices

APPENDIX – 1
ESTIMATION OF LIGNIN
(GOERING AND VANSOEST, 1975)

Principle

Refluxing the sample material with acid detergent solution which removes the water soluble and materials other than the fibrous component. The left-out material is weighed after filtration, dried, treated with 72% H_2SO_4 and filtered, dried and ashes. The loss of weight on ignition gives the acid detergent lignin.

Reagents

- Acid Detergent Solution
- Dissolve 20 g of acetyl trimethyl ammonium bromide in one litre of 1 N sulphuric acid.
- 72% H_2SO_4 (W/V)
- Acetone
- Round Bottom Flask and Refluxing Set
- Muffle Furnace
- Sintered Glass Crucible – G2

Procedure

A. Acid Detergent Fibre (ADF)

- 1g of powdered sample and 100ml of acid detergent solution was placed in a round bottom flask and boiled for 5 – 10 minutes. The heat was reduced to avoid foaming as boiling begins. Refluxing was done for 1 hour after the onset of boiling. Boiling was adjusted to slow or even level.
- The container was removed, swirled and filtered the contents through a pre-weighed sintered glass crucible (G2) by suction and washed with hot water twice.
- Then, washed with acetone and break up the lumps. Acetone washing was repeated until the filtrate was colourless.

- Dried at 100°C for overnight.
- Weighed after cooling in a desiccator.
- ADF content was expressed in percentage i.e., W/S x 100, Where W is the weight of the fibre and S is the weight of the sample.

B. Determination of Acid Detergent Lignin (ADL)

- ❖ ADF was transferred to a 100 ml beaker with 25 - 50 ml of 72% sulphuric acid. 1g of asbestos was added to it. It was allowed to stand for 3 hrs with an intermittent stirring with a glass rod.
- ❖ The acid was diluted with distilled water and filtered with preweighed Whatman No. 1 filter paper. The glass rod and the residue were washed several times to get rid of the acid.
- ❖ The filter paper was dried at 100°C and weighed after cooling in a desiccator.
- ❖ The filter paper was transferred to a preweighed silica crucible and ashed the filter paper with the content in a muffle furnace at 55°C for about 3h.
- ❖ The crucible was cooled in a desiccator and weighed. The ash content was calculated.
- ❖ 1 g asbestos was taken as blank and then added 72% H_2SO_4 and followed the steps from 2 - 5.

Calculation

$$\text{ADL (\%)} = \frac{\dfrac{\text{Weight 72\% } H_2SO_4 \text{ washed fibre}}{\text{(Test – Asbestos blank)}} - \dfrac{\text{Ash}}{\text{(Test – Asbestos blank)}}}{\text{Weight of sample}} \times 100$$

APPENDIX – 2

ESTIMATION OF CELLULOSE

(UPDEGROFF, 1969)

Principle

Cellulose undergoes acetolysis with acetic/nitric reagent forming acetylated cello dextrins which get dissolved and hydrolyzed to formed glucose molecules upon treatment with 67% H_2SO_4. This glucose molecule is dehydrated to form hydroxyl methyl furfural which forms green coloured product with anthrone and the colour intensity is measured at 630 nm.

Reagents

- Acetic/Nitric reagent: 150 ml of 80% 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 two hrs before use.
- 67% sulphuric acid.

Procedure

A quantity of 0.1g of sample was taken in a test tube, 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 discarded. The residue was washed with distilled water and 10 ml of 67% sulphuric acid was added and allowed to stand for 1 hr.1ml of the solution was taken and diluted to 100ml.From the above diluted solution, 1ml was taken, to which 10ml 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 sample was calculated using a standard graph corresponding to 40 - 200 µg of cellulose.

APPENDIX – 3

ESTIMATION OF ORGANIC CARBON
WET CHROMIC ACID OXIDATION METHOD
(WALKLEY AND BLACK, 1934)

Principle

Organic carbon present in organic matter is oxidised by chromic acid in the presence of conc. H_2SO_4. Potassium dichromate on reaction of H_2SO_4 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 chromic acid left unused by the organic matter is determine by back titration with 0.5 N ferrous sulphate or ferrous ammonium sulphate using diphenylamine indicator.

Reagents

- 1 N potassium dichromate: Exactly 49.04 g of $K_2Cr_2O_7$ was dissolve in one litre of distilled water.

- Diphenylamine indicator: 0.5 g diphenylamine was dissolved in 20 ml of water and 100 ml of Conc. H_2SO_4 was added.
- 0.5 N ferrous sulphate or ferrous ammonium sulphate: 139.0 g of ferrous sulphate or 196 g of ferrous ammonium sulphate was dissolved in 800 ml of distilled water. 20 ml of Conc. H_2SO_4 was added and the volume was made up to one litre.
- Conc. H_2SO_4
- Phosphoric acid (Orthophosphoric acid 85%).

Procedure

Exactly 0.5gm of soil (passed through 0.2 mm sieve) was weighed and transferred to 500 ml conical flask. 10ml of 1N $K_2Cr_2O_7$ was added and mixed well by swirling the flask. Added 20ml of conc. H_2SO_4 mixed by gentle rotation for one minute to ensure complete contact of the reagent with the soil. Allowed the contents to stand for 20-30 minutes. Kept the flask on asbestos sheet to avoid burning of table due to intense heat. Added 200ml of water after 30 minutes. Then added 10 ml of phosphoric acid and 1 ml of diphenylamine indicator. Titrated the solution with 0.5N ferrous ammonium sulphate. As the titration proceeds the dull green colour shifted to the turbid blue and at the end point bright green colour developed. Conducted simultaneously a blank titration (without soil) and the volume of 0.5N ferrous ammonium sulphate consumed was noted.

Calculation

Weight of soil taken = 0.5g

Volume of 1N $K_2Cr_2O_7$ = 10ml

Volume of 0.5N ferrous ammonium sulphate
used for blank titration = X ml (Sample T. V)

Volume of 0.5N ferrous ammonium sulphate
used for blank titration = Y ml (Sample T. V)

Xml of $FeSO_4$ reduces 10ml of 1N $K_2Cr_2O_7$

Therefore, Y ml of $FeSO_4$ reduces Y/X* 10ml

Hence actual quantity of 1N $K_2Cr_2O_7$ used for
oxidation of organic matter = 10 - (10 * Y/X)

1ml of 1N $K_2Cr_2O_7$= 0.003gm of 'C'

Therefore 10-(10*Y/X) ml of 1N $K_2Cr_2O_7$ = 10 – (10 * Y/X) * 0.003

This is present in 0.5gm of soil

Therefore in 100gm = 10 - (10*Y/X) * 0.003 * 100/0.5

Organic matter (surface soil) = organic carbon * 1.724

Organic matter (sub surface soil) = organic carbon * 2.5

APPENDIX - 4
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 concentrated sulphuric acid and concentrated perchloric acid.
- Mixed indicator: 0.5g bromocresol green and 1g of methyl red were dissolved in 100ml of 90% ethyl alcohol.
- 40% sodium hydroxide solution.
- 2% boric acid.
- Concentrated sulphuric acid (0.02 N).

Procedure

- A quantity of 0.2g of dried, sieved and homogenized sample was taken in a micro kjeldhal digestion flask (50ml capacity), to which, 12ml of diacid was added.
- Complete digestion was ensured by adding one drop of perchloric acid and the contents turns colourless like water.
- The volume was made upto 100ml with distilled water.
- 10ml aliquot was pipette out into a Wagnor- Parnas distillation apparatus and 10ml of 2% boric acid with mixed indicator was kept in a beaker at the delivery end of the distillation apparatus.
- To the distillation apparatus, 10ml of 40% 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 0.02 N sulphuric acid until a red colour was appeared.

Total nitrogen content of the sample was determined by the formula.

$$\text{Total nitrogen (\%)} = \frac{0.00028 \text{ x T.V. x } 100 \text{ x } 100}{10 \text{ x } 0.2}$$

Where,

T.V.	=	Titre value
0.00028	=	1 ml of 0.02 N sulphuric acid utilized
10	=	Volume of extract taken for distillation
0.2	=	Weight of sample (g)
100	=	Total volume (ml)

APPENDIX - 5
ESTIMATION OF TOTAL PHOSPHORUS
(JACKSON, 1973)

Principle

Phosphorus is precipitated as ammonium phosphomolybdate in nitric acid medium. The precipitate is filtered, washed free of acid, dissolved in a known excess of standard alkali and the excess alkali is determined by back titration with a standard acid using phenolphthalein indicator.

Reagent

- Hydrochloric acid – 1:1
- Nitric acid – 1:1
- Nitric acid – 1:1
- Conc. ammonium hydroxide
- Conc. nitric acid
- Solid ammonium nitrate
- Ammonium molybdate solution – 20 percent
- Potassium hydroxide – 0.1619N
- Nitric acid - 0.1619N
- Phenolphthalein

Procedure

- 200 ml of HCl extract of the sample was pipette out into a 400 ml beaker and evaporated to a small bulk.
- Then, it was transferred to a silica basin using hot water and evaporated to dryness over a water bath.
- The silica basin was kept in an air oven at 105 to 110 ^{o} C for 3 h to dehydrate the silica.
- This residue was dissolved by adding a small quantity of 1:1 hydrochloric acid and evaporated to dryness over a water bath.
- The residue was again dissolved in nitric acid, adding sufficient amount of nitric acid, to dissolve the same.
- The insoluble silica was allowed to settle overnight and then filtered through No. 42 filter paper and the residue was washed in the silica basin and on the filter paper with small quantities of 1:4 nitric acid till no yellow colour was left either in the basin or in the filter paper. The filtrate was collected in a 250 ml beaker.
- The extract was made alkaline with conc. ammonium hydroxide.
- To this, 5g of solid ammonium nitrate was added and kept on a thermostat at 65 ^{o} C for 15 minutes.
- The precipitant mixture was prepared by taking 7 ml of conc. nitric acid and 3 ml of distilled water in a 100 ml beaker and 10 ml of 20 percent ammonium molybdate was added to this solution drop by drop with constant stirring.
- 10 ml of this precipitant mixture was added drop by drop to the beaker in the thermostat with constant stirring and kept in the thermostat for another half an hour at 65 ^{o} C and allowed the precipitate to settle well.
- Then, it was filtered through No.40 filter paper by decantation, pouring only the supernatant liquid to the filter paper.
- The precipitate was then washed with cold distilled water till the filtrate runs free of acid.
- The filter paper was then transferred with the precipitate to the same beaker in which precipitation was done and enough water was added to make the filter paper into a pulp.
- Now, 0.1619 N KOH was added from the burette, till the yellow precipitate was completely dissolved leaving a colourless solution. Then, another 5 ml of 0.1619N KOH was added to keep the alkali in fair excess quantity.

- A drop of phenolphthalein was added and the excess alkali was titrated against 0.1619 N nitric acid. Disappearance of pink colour indicated the end point.

Calculation

Weight of sample taken = W g

Volume of HCl extract prepared = 500 ml

Volume of HCl extract pipette out for analysis = 200 ml

Volume of 0.1619N KOH added in excess = a ml

Volume of 0.1619N HNO_3 used for back titration =b ml

Therefore, actual volume of 0.1619N KOH

used to dissolve the precipitate = (a-b)

1 ml of 0.1619N KOH = 0.0005 gm P2O5

(a-b) ml of 0.1619N KOH = 0.0005 x (a-b) x gm P2O5

This was present in 200 ml of HCl extract

Therefore, in 500 ml = 0.0005 x (a-b) x 500/200

This was present in W gm of sample

Therefore, in 100 gm = 0.0005 x (a-b) x 500/200 x 100/W

Percentage of P_2O_5 on moisture free basis

= 0.0005 x (a-b) x 500/200 x 100/W x 100/(100 – M)

(M – Moisture content of the sample

APPENDIX - 6
ESTIMATION OF TOTAL POTASSIUM
FLAME PHOTOMETER METHOD
(JACKSON, 1973)

Principle

Certain elements when excited in flame, emit radiation. The excitation causes one of the outer electrons of neutral atoms to jump to an outer orbit of higher energy level or the atoms may be excited sufficiently to loose an electron completely. When excited atoms return to lower energy levels, light of characteristics wavelength is emitted. The flame photometer measures this radiation intensity which is proportional to the concentration in a solution.

Preparation

1.907g of KCl was dissolved in 1 litre of distilled water (1000 ppm of K). From this, various standards were prepared ranging from 10 to 100ppm.

Procedure

- The atomizer was fixed in its place and introduced with distilled water.
- The compressor was started and the air pressure was adjusted to 10 psi.
- The gas was opened to light the burner through the window. Flow of gas was adjusted to give a central bluish cone.
- Zero was set with distilled water by using the zero-adjustment knob. Then, 100 ppm K solution was introduced and adjusted to read 100 on the scale. Again, distilled water was introduced and adjusted to zero.
- This process was repeated till the metre reading showed zero with distilled water and 100 with 100 ppm solution without zero adjustment.
- Then, various standard solutions were introduced, the readings were recorded and the standard curve was drawn.
- The filtrate was taken from sesquioxide estimation in a small vial and introduced through the atomizer. The readings were recorded and the percentage of K was calculated by using the standard curve.

Calculation

Weight of sample taken	= W g
Volume of HCl extract prepared	= 500 ml
Volume HCl extract pipette out	
for sesquioxide estimation	= 50 ml
Volume of sesquioxide filtrate made up to	= 250 ml
Metre reading	= G
Equivalent ppm from standard curve	= A
i.e. 1 ml of the solution contains	
A microgram of K	= A/106 g of K
Therefore, in 250 ml of the solution	= A/106 x 250
This was present in 50 ml of HCl extract	
Therefore, in 500 ml	= A/106 x 250 x 500/50 g
This was present in W gm of sample	

Therefore, in 100 gm = A/106 x 250 x 500/50 x 100/W g

Percentage of K on moisture free basis

= A/106 x 250 x 500/50 x 100/W x 100/(100 – M)

(M – Moisture content of sample)

APPENDIX- 7

ESTIMATION OF CALCIUM AND MAGNESIUM

VERSANATE METHOD

(JACKSON, 1973)

Principle

Calcium and magnesium get complexed by EDTA in the order calcium first followed by magnesium. Calcium is estimated first by using murexide indicator at pH 12 in the presence of sodium hydroxide. Then calcium and magnesium is estimated using Eriochrome Black – T at pH 10 in the presence of ammonium chloride and ammonium hydroxide buffer solution.

Reagents

- 0.02 N EDTA
- 10% sodium hydroxide
- Ammonium chloride – ammonium hydroxide buffer solution
- Murexide solution
- Eriochrome Black – T indicator

Procedure

Calcium alone

- Pipette out 10 ml of sesquioxide filtrate into a porcelain basin.
- Add 10% sodium hydroxide solution drop by drop to neutralise the activity (red litmus turns blue) and another 5ml excess to maintain the pH at 12.
- Add a pinch (50 mg) of murexide indicator and titrate with 0.02N EDTA till the colour changes from pinkish red to purple or violet.

Calcium and Magnesium

- Pipette out 10 ml of seaquioxide filterate into a porcelain basin.
- Add ammonium chloride – ammonium hydroxide buffer solution drop by drop to neutralise the acidity (use red litmus paper) and 5 ml excess to maintain the pH at 10.

- Add 2 – 3 drop of Eriochrome Black – T indicator solution and titrate with 0.02 N EDTA till the colour changes from purple red to sky blue.

Calculation

Weight of the sample taken	= W g
Volume of hydrochloric acid extract prepared	= 500 ml
Volume of hydrochloric acid extract pipette out for R_2O_3 estimation	= 50 ml
Volume of R_2O_3 filtrate made upto	= 250 ml
Volume of R_2O_3 filtrate pipetted out for calcium estimation	= 10 ml
Volume of 0.02 N EDTA used for calcium and magnesium	= a ml
Volume of 0.02 N EDTA used for calcium alone	= b ml
Volume of 0.02 N EDTA used for magnesium alone	= (a-b) ml
1 ml of 0.02 N EDTA	= 0.0004 g calcium
1 ml of 0.02 N EDTA	= 0.0004 g magnesium

Percentage of calcium on moisture free basis

$$= 0.0004 * \mathrm{b} * \frac{250}{10} * \frac{500}{50} * \frac{100}{\mathrm{W}} * \frac{100}{(100 - \mathrm{M})}$$

Percentage of calcium on moisture free basis

$$= 0.00024 * (\mathrm{a} - \mathrm{b}) * \frac{250}{10} * \frac{500}{50} * \frac{100}{\mathrm{W}} * \frac{100}{(100 - \mathrm{M})}$$

M= Moisture basis

APPENDIX- 8

PROTEIN

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 tyrosine and tryptophan present in the protein plus the colour developed by the biuret reaction of the protein with the alkaline cupric tartrate are measured in the Lowry's method.

Materials

- 2 % sodium carbonate in 0.1 N sodium hydroxide (Reagent A).
- 0.5 % copper sulphate ($CuSO_4$. $5H_2O$) in 1% potassium sodium tartrate (Reagent B).

 Alkaline copper solution: 50 ml of reagent A and 1ml of reagent B were mixed prior to use (Reagent C).

 Folin-Ciocalteau reagent (Reagent D).

 Protein solution (stock standard): Weighed accurately 50mg of bovine serum albumin (fraction V) and dissolved in distilled water and made up to 50 ml in a standard flask.

Working standard

10 ml of the stock solution was diluted to 50 ml with distilled water in a standard flask. 1ml of this solution contains 200 µg protein.

Procedure

Extraction of Protein from Sample

Extraction is carried out with buffers used for the enzyme assay. About 50mg of the sample was taken and 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

A quantity of 0.2, 0.4, 0.6, 0.8 and 1ml of aliquots of the working standard were pipetted into a series of test tubes 0.1ml and 0.2ml of the sample extract in two other test tubes. The volume was made up to 1ml in all test tubes. A test tube with 1ml of water served as the blank. 5ml of reagent C was added to each tube including the blank, mixed well and allowed to stand for 10minutes. Then, 0.5ml of reagent D was added, mixed well and incubated at room temperature in the dark for 30 minutes. Blue colour developed was read in a spectrophotometer (UV-vis Spectrophotometer model 108, Systronics, India). A standard graph was drawn and the amount of protein in the sample was calculated

Calculation

Expressed the amount of protein mg/gm or 100gm sample.

$$\frac{\text{mg of protein}}{\text{Volume of test standard}} \times \text{concentration of the standard}$$

APPENDIX - 9

CARBOHYDRATE

ESTIMATION OF CARBOHYDRATE (HEDGE AND HOFREITER, 1962)

Anthrone method

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 colour in a dilute solution and a blue color in a concentrated solution. This compound forms a green colored product with an absorption maximum at 630 nm.

Materials

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

Procedure

100mg of the sample (leaf) was taken in a boiling tube with 5ml 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 100ml and centrifuged. The supernatant was collected and 0.5 and 1ml 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 1ml and '0' served as blank. The volume was made up to 1ml in all the test tubes including the sample test tubes by adding distilled water. Then, 4ml 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 630nm. 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.

$$\frac{\text{Mg of glucose}}{\text{Volume of test sample}} \times 100$$

APPENDIX - 10

CHLOROPHYLL

ESTIMATION OF CHLOROPHYLL (ARNON, 1949)

Principle

Chlorophyll was extracted in 80% acetone. The absorption at 663 nm, 645 nm and 652nm were read in a spectrophotometer using the absorption coefficients and the amount of chlorophyll content were calculated.

Materials

Analytical grade acetone was diluted to 80 % acetone (pre chilled)

Procedure

Accurately weighed 1g of finely cut and well mixed leaf sample was ground to a fine pulp with the addition of 20ml of 80% acetone with a mortar and pestle and was centrifuged as 5,000 rpm for 5 minutes. The supernatant was transferred to a 100ml volumetric flask. The residue was ground with 20ml of 80% 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% acetone and the washing was collected in the volumetric flask. The volume was made up to 100ml with 80% acetone. The absorbance of the solution was read at 645, 663 and 652 nm against the solvent (80% acetone) blank.

Calculation

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

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

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

$$\text{(iii) Total chlorophyll mg g} - 1 \text{ tissue } 20.2\,A\,(645) + 8.02\,A\,(663) \times \frac{V}{1000 \times W}$$

Where,

A	=	absorbance of specific wavelengths
V	=	final volume of chlorophyll extract in 80% acetone.
W	=	fresh weight of tissue extract

APPENDIX- 11
CRUDE PROTEIN
ESTIMATION OF CRUDE PROTEIN CONTENT
(AOAC, 2016)

Reagents

- Conc. Sulphuric acid,
- Digestion mixture: 10 parts Potassium sulphate + 1 parts Copper sulphate, 40 per cent Sodium hydroxide
- 0.1N Hydrochloric acid
- 0.1N Sodium hydroxide, Methyl red indicator

Micro Kjeldahl method was adopted to determine the percentage of nitrogen content and a conversion factor of 6.25 was used to calculate crude protein content.

Digestion of Samples

Weighed sample (0.5g of moisture-free ground adzuki bean seed sample) was digested with conc. sulphuric acid (20ml) and 5g of digestion mixture (containing mixture of potassium sulphate and copper sulphate in the ratio of 10:1) in a Kjeldahl digestion flask, till the contents became free from organic carbon and appeared as a clear solution. After cooling the content were diluted with small amount of distilled water then transferred into a 50ml volumetric flask and volume made up to 50ml with distilled water.

Distillation

An aliquot of (10ml) digested sample was transferred to a distillation assembly unit followed by addition of 10ml of 40 per cent sodium hydroxide solution. During distillation, ammonia liberated was collected in a 100ml conical flask containing 10ml of 0.1N hydrochloric acid to which methyl red indicator (2-3 drops) was added.

Titration

The excess of acid in the receiver was back titrated against 0.1N sodium hydroxide and the amount of sodium hydroxide used was recorded. One blank (containing conc. sulphuric acid and digestion mixture) was similarly digested and distilled.By this method the per cent nitrogen present in the sample was calculated which was then multiplied by the conversion factor 6.25 (for pulses) to get the crude protein as follows

Crude Protein (%) =

$$\frac{(\text{Sample titre value - Blank titre value}) \times 0.0014 \times \text{Total volume} \times 100 \times 6.25}{\text{Aliquot used} \times \text{Weight of sample (g)}}$$

APPENDIX- 12

LEGHAEMOGLOBIN

ESTIMATION OF LEGHAEMOGLOBIN CONTENT (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: Sodium (0.1 M) / Potassium phosphate buffer (pH 7.4).
- Alkaline pyridine reagent: Dissolved 0.8 g NaOH in 50 ml water and cool. Added 33.8 ml of pyridine (33.2g), dissolved and diluted to 100 ml with water. This produces 4.2 M pyridine in 0.2 M NaOH.
- Sodium Dithionate: Ground finely and stored in small stopped tubes in dessicator. Potassium Hexacyanoferrate.

Procedure

Extraction: Fresh 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 was discarded. The turbid reddish-brown filtrate was clarified by centrifuging at 10,000 rpm for 10-30 minutes diluted suitably. To a suitable volume (2-5 ml) of the extract, an equal volume 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 dithionate 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) = A556 – A539 x 2D/23.4

Where, D is the initial dilution.

(The calculation is based upon the equation E = 23.4 x 103 mol^{-1} cm^{-1})

APPENDIX – 13

ESTIMATION OF AVAILABLE NITROGEN IN SOIL

ALKALINE PERMANGANATE METHOD

(SUBBIAH AND ASIJIA, 1956)

Principle

A known weight of soil is mixed with excess of alkaline permanganate and distilled organic matter present in soil is oxidised by the nascent oxygen liberated by $KMnO_4$ in the presence of NaOH and thus ammonia is released. This released ammonia is absorbed in a known volume of boric acid (2%) containing double indicator and converted to ammonium borate. This ammonium borate is titrated against standard H_2SO_4.

Reagents

- 0.32% $KMnO_4$ solution (3.2 gm of $KMnO_4$ dissolved in one litre of distilled water).
- 2.5% NaOH solution (25 gm of NaOH dissolved in one litre of distilled water).
- 2% boric acid (20 gm of boric acid dissolved in one litre of distilled water).
- N/50 H_2SO_4 (30 ml of Conc. H_2SO_4 is diluted to one litre with distilled water and standardized by titration with N/10 Na_2CO_3. This gives N/10 H_2SO_4. From this N/50 H_2SO_4 is prepared by dilution.
- Double indicator bromocresol green (0.5 gm) and methyl red (0.1 g) dissolved in 100 ml and ethyl alcohol.

Procedure

Weighed 20 gm of soil and transferred into a distillation flask. Added 30 ml of distilled water to moist the soil and 1 ml of liquid paraffin. Added few pieces of glass beads to avoid frothing. Added 100 ml of freshly prepared 0.32% $KMnO_4$ and 100 ml 2.5% NaOH to the soil in the distillation flask. A 100 ml beaker containing approximately 20 ml of 2% boric acid with double indicator was kept below the delivery end of the condenser in the distillation set. Distilled the contents and the liberated ammonia was collected in boric acid. Distillation continued until the release of ammonia. Titrate the ammonia collected in boric acid with N/50 H_2SO_4.

Calculation

Weight of the soil = 20 g

Volume of N/50 H_2SO_4	= X ml (titre value)
1ml of N/10 H_2SO_4	= 0.0014 g N
Therefore, 1 ml of N/50 H_2SO_4	= 0.00028 gm N
X ml of N/50 H2SO4	= 0.00028 * X g N
This is present in 20 gm of soil	
Therefore, N present in Kg/Ha	= 0.00028 (X/20) * 10^6

APPENDIX - 14

ESTIMATION OF AVAILABLE PHOSPHORUS IN SOIL

CALORIMETRY METHOD

(BRAY 1 METHOD – JACKSON, 1973)

Principle

The combination of HCl and NH_4F extracts acid soluble forms of phosphorus such as mono calcium phosphate. The flouride ion has the special property of complexing Al^{+++} and Fe^{+++} in acid solution with consequent release of phosphorus held in the soil by these ions. The phosphorus so released into the soil solution in estimated calorimetrically as available phosphorus.

Reagents

- NH_4F solution (1N): 37g of NH_4F was dissolved in 1 litre of distilled water.
- HCl (0.05N): 20.2 ml conc. HCl diluted 500 ml with distilled 500 ml with distilled water.
- Bray No. 1 extractant [0.03 NH_4F and 0.02 N HCl]: 15 ml of 1N NH_4F and 25 ml of 0.5N HCl are mixed and the volume was upto 500 ml with distilled water.
- Ascorbic acid.

Procedure

Weighed 5gof soil and transfer to a 100 ml polythene shaking bottle. Added 50 ml of Bray 1 extractant. Shake the contents in a reciprocatory mechanical shaker for one minute. Filtered the contents through whatman No. 40 filter paper. Simultaneously conducted a blank. Pipetted out 5 ml of filterate into 25 ml volumetric flask. Added 4 ml of reagent B as in Olsen's method and made up the volume to 25 ml. The intensity of the colour developed was measured in a photoelectric calorimeter using filter (660 nm).

Calculation

Weight of soil taken = 5 g

Volume of NaHCO3 = 50 ml

Volume of extractant solution used for

Phosphorus estimation (aliqreo) = 5 ml

Calorimeter reading = T

Concentration of phosphorus read from

Standard graph for the reading T = X ppm

= X mg/ml

= X/106 gm/ml

Therefore, in 25 ml solution $= X/10^6 * 25$ g

This is present in 50 ml of extractant solution and 5g of soil

Therefore, available P_2O_5 in Kg/Ha $= X * 25 * 50 * 2 * 10^6$

$10^6 * 5 * 5$

APPENDIX – 15

ESTIMATION OF AVAILABLE POTASSIUM IN SOIL

FLAME PHOTOMETRY METHOD

(STANDFORD AND ENGLISH, 1949)

Principle

The potassium ions in the exchange site are replaced with NH_4+ and K+ which is released. The concentration of K ions in the solution is then determined using flame photometer.

Reagents

1 N Ammonium acetate (Neutral in pH): Dissolved 77 g of AR grade ammonium acetate in 1000 ml distilled water. pH adjusted to 7.0.

Procedure

Transferred 5g of soil into a polythene shaking bottle. Added 25 ml of 1 N ammonium acetate and contents shaked in a mechanical reciprocating shaker for 5 minutes. Contents filtered through whatman No. 40 filter paper. Filtrates were fed into the flame photometer and the readings recorded. Using standard curve available potassium content was calculated.

Calculation

Weight of the soil taken = 5 g

Volume of the extractant used = 25 ml

Flame photometer reading = T

Concentration of K in the standard curve = X ppm

= X mg/ml

= $X/10^6$ gm/ml

Therefore in 25 ml solution = $X/10^6*25$g

This is present in 5gm of soil

Therefore available K in soil in kg/ha = $X/10^6*25*2*10^6/5$

APPENDIX- 16

ANTIOXIDANT ACTIVITY

HYDROGEN PEROXIDE SCAVENGING ACTIVITY (RUCH *et al.*, 1989)

Principle

The UV absorption of H_2O_2 can be easily detected at 230 nm. On scavenging of H_2O_2 by the plant extract, the absorption decreases at this wavelength. This characteristic is used to measure their H2O2 scavenging efficiency.

Reagents

1. Phosphate buffer (0.1M, pH 7.4)
2. H_2O_2 (40mM) in phosphate buffer

Procedure

- A solution of H_2O_2 (40mM) was prepared using phosphate buffer.
- Plant extracts at the concentration of 5µl were added to H_2O_2 solution (0.6ml) and the volume was made up to 3ml.
- The reaction of the mixture was recorded at 230 nm absorbance in a spectrophotometer.
- A solution containing on phosphate buffer without H_2O_2, was prepared as blank.

The H_2O_2 scavenging of the plant extracts was calculated as

$$\% \text{ scavenging of hydrogen peroxide} = \frac{A0 - A1 \times 100}{A0}$$

A_0 - Absorbance of control; A_1 - Absorbance in the presence of plant extracts

APPENDIX- 17

ANTIOXIDANT ACTIVITY

REDUCING POWER ASSAY (OYAIZU, 1986)

Principle

Substances, which have reduction potential, react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}), and then reacts with ferric chloride to form ferric ferrous complex that has a maximum absorption at 700 nm.

Potassium ferricyanide + Ferric chloride $\longrightarrow$ Potassium ferrocyanide + Ferrous chloride

Reagents

1. Potassium ferricyanide (1%)

2. Phosphate buffer (0.2 M, pH 6.6),

3. Trichloro acetic acid (10%)

4. Ferric chloride (0.1%)

5. Ascorbic acid (1%)

Procedure

- 0.5 ml of the plant extracts were mixed with phosphate buffer (2.5 ml) and potassium ferricyanide (2.5 ml).
- This mixture was kept at 50ºC in water bath for 20 minutes. After cooling, 2.5 ml of 10% trichloro acetic acid was added and centrifuged at 3000 rpm for 10 minutes whenever necessary.
- The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and a freshly prepared ferric chloride solution (0.5 ml).
- The absorbance was measured at 700 nm.

APPENDIX - 18

ANTIOXIDANT ACTIVITY

NITRIC OXIDE RADICAL SCAVENGING ACTIVITY (GREEN *et al.,* 1982)

Principle

At physiological pH, sodium nitroprusside generates nitric oxide which interacts with O_2 to produce nitrite ions, which is measured at 546 nm.

Reagent

1. Sodium nitroprusside (100 mM),

2. pH buffers saline (PBS) pH 7.4,

3. Griess reagents

Procedure

Sodium nitroprusside (2 ml), phosphate buffered saline (0.5ml) and plant extract (0.5µl) were mixed and incubated at 25°C for 30 minutes. Griess reagent (0.5 ml) was added and allowed to stand for another 30 minutes. The pink colour chromophore was developed and the absorbance was read at 546 nm.

APPENDIX- 19

ANTIOXIDANT ACTIVITY

DPPH RADICAL SCAVENGING ACTIVITY (MENSOR *et al.,* 2001)

Principle

DPPH radical reacts with an antioxidant compound that can donate hydrogen, and gets reduced. DPPH, when acted upon by an antioxidant, is converted into diphenylpicryl hydrazine. This can be identified by the conversion of purple to light yellow colour.

Reagents

1. DPPH - 2, 2-diphenyl-2-picryl hydrazyl hydrate (0.3mM in methanol)
2. Methanol

Procedure

- The extracts (20µl) were added to 0.5ml of methanolic solution of DPPH and 0.48ml of methanol.
- The mixture was allowed to react at room temperature for 30 minutes.
- Methanol served as the blank and DPPH in methanol, without the extracts, served as the positive control.
- After 30 minutes of incubation, the discolouration of the purple colour was measured at 518nm in a spectrophotometer.

The radical scavenging activity was calculated as follows

$$\text{Scavenging activity \%} = \frac{\text{Control} - \text{Sample}}{\text{Control}} \times 100$$

APPENDIX - 20

ANTIBACTERIAL ACTIVITY

ANTIBACTERIAL METHOD (BAUER *et al.,* 1996).

Pure culture of bacterial source

The selected microorganisms were cultured as pure culture in agar slant by using nutrient agar medium

Nutrient Agar medium

Beef extract – 3 g

Peptone – 5 g

Agar –1 5 g

Distilled water – 1000 ml

pH – 7.0 ± 0.2

The above ingredients (except agar) were dissolved separately in 200 ml of distilled water. The agar was dissolved in 250 ml of warm distilled water and make up to 1000 ml. pH of the medium was adjusted to 7.0 ± 0.2, sterilized in an autoclave or pressure cooker and used for further analysis.

Slant Culture method

The nutrient agar medium was poured in sterilized culture tubes in aseptic condition using laminar airflow chamber. Then the tubes were placed in slanting position. After the solidification of medium, the bacterial cultures were inoculated separately in zig zag pattern on the surface of the medium using inoculation loop in an aseptic condition. The tubes were incubated at 37°C for 24 h.

Broth culture

The selected microbes were also inoculated in the conical flask containing nutrient broth.

Nutrient broth

Beef Extract - 3 g

Peptone - 5 g

Distilled water - 1000 ml

pH - 7.0 ± 0.2

The above ingredients was weighed and dissolved in 1000 ml of distilled water. pH of the broth was adjusted to 7. 0± 0.2 and then it was sterilized in an autoclave for 30 minutes. The medium was used for further experiment.

Suspension Culture

10 ml of sterile nutrient broth was taken in an sterilized culture tube under aseptic condition. The test organisms were inoculated separately through inoculation loop, the inoculated tubes were kept in an incubator at 37°C and it was used for antibacterial activity.

Maintenance of Culture

Bacterial cultures were streaked on nutrient agar to isolate and obtain the pure culture. Then the single pure culture colony was streaked and maintained on nutrient agar medium, stored at 37°C to keep the strains viable. They were characterized by using biochemical tests.

Assessment of antibacterial activity (well diffusion method)

After the characterization of selected microbes, the selected plant seeds were analyzed for the antibacterial activity by measuring the diameter of zone of inhibition around the well by agar well diffusion method (Bauer *et al.,* 1996). The antibacterial activity was assessed in the petri plate using muller hinton agar medium.

Muller Hinton Agar Medium

Beef Extract - 20 g
Starch - 1.5 g
Peptone - 17.5 g
Agar - 17 g
Distilled water - 1000 ml
pH - 7.0 ± 0.2

The above ingredients (except agar) were dissolved in 500 ml of distilled water. Agar was separately dissolved in 300 ml of warm distilled water and it was made up to 1000 ml. The pH of the medium was adjusted to 7.0 ± 0.2, sterilized in an autoclave for 30 minutes. The prepared muller hinton agar medium was used for antibacterial activity. The antibacterial activity was carried out by using the solvents of aqueous and methanol extracts at 150µl concentration in the selected plants against the selected pathogen. The muller hinton agar medium was poured in a sterilized petri plates and then it was allowed for solidification. The selected microorganisms of *Escherichia coli and Staphylococcus aureus* were swabbed over

the medium using swab culture technique in separate plates. After swabbing, five wells were prepared in the medium using sterile cork borer (5mm) carefully without damaging the agar. The five wells were loaded with respective solvent and standard antibiotic. Likewise separate plates were prepared with separate wells for the selected plants against selected microorganism. Later the plates were incubated at 37°C for 24 h. After 24 h, the diameter of the zone of inhibition was measured in mm using scale.

Annexure - I

PLANT AUTHENTICATION CERTIFICATE

Vigna mungo (L) Hepper

भारतसरकार
GOVERNMENT OF INDIA
पर्यावरण,वनऔरजलवायुपरिवर्तनमंत्रालय
MINISTRY OF ENVIRONMENT, FOREST & CLIMATE CHANGE
भारतीयवनस्पतिसर्वेक्षण
BOTANICAL SURVEY OF INDIA

दक्षिणीक्षेत्रीयकेन्द्र / Southern Regional Centre
टी.एन.ए.यूकैम्पस/ T.N.A.U. Campus
लाउलीरोड/ Lawley Road
कोयंबत्तूर/ Coimbatore - 641 003

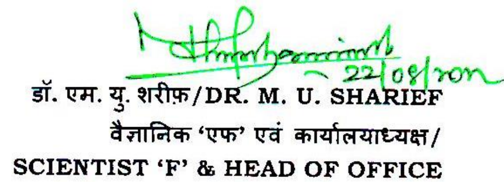
टेलीफोन / Phone: 0422-2432788, 2432123, 2432487
टेलीफक्स/ Telefax: 0422- 2432835
ई-मैल/E-mail id: sc@bsi.gov.in
bsisc@rediffmail.com

सं. भा.व.स./द.क्षे.के./No.: BSI/SRC/5/23/2022/Tech 1401 दिनांक/Date: 22nd August 2022

पादप प्रमाणीकरण प्रमाणपत्र / PLANT AUTHENTICATION CERTIFICATE

The plant specimen given by you for authentication is identified as ***Vigna mungo* (L.) Hepper - FABACEAE.**

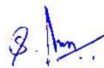
अभिनिर्धारित प्रतिरूप को संबंधित कॉलेज/विभाग/संस्थान के पादपालय में परिरक्षण हेतु वापस किया जाता है। / The identified specimen is returned herewith for preservation in their College/ Department/ Institution Herbarium.



डॉ. एम. यु. शरीफ़/DR. M. U. SHARIEF
वैज्ञानिक 'एफ' एवं कार्यालयाध्यक्ष/
SCIENTIST 'F' & HEAD OF OFFICE

सेवा में / To

Ms. PINKY RAIHING
Ph.D. Research Scholar
Department of Botany
Avinashilingam Institute for Home Science &
Higher Education for Women
COIMBATORE – 641 043



Annexure - II

PLANT AUTHENTICATION CERTIFICATE

Lablab purpureus **(L) Sweet**

भारतसरकार
GOVERNMENT OF INDIA
पर्यावरण, वन और जलवायु परिवर्तन मंत्रालय
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भारतीय वनस्पति सर्वेक्षण
BOTANICAL SURVEY OF INDIA

दक्षिणीक्षेत्रीयकेन्द्र / Southern Regional Centre
टी.एन.ए.यूकैम्पस/ T.N.A.U. Campus
लाउलीरोड/ Lawley Road
कोयंबत्तूर/ Coimbatore - 641 003

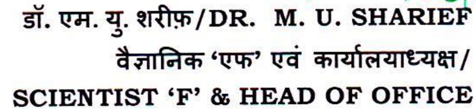
टेलीफोन / Phone: 0422-2432788, 2432123
टेलीफक्स/ Telefax: 0422- 2432835
ई-मैल/E-mail id: sc@bsi.gov.in
bsisc@rediffmail.com

सं. भा.व.स./द.क्षे.के./No.: BSI/SRC/5/23/2022/Tech. 1189

दिनांक/Date: 9th June 2022

पौधे प्रमाणीकरण प्रमाणपत्र / PLANT AUTHENTICATION CERTIFICATE

The plant specimen brought by you for authentication is identified as *Lablab purpureus* (L.) Sweet - FABACEAE. The identified specimen is returned herewith for preservation in their College/ Department/ Institution Herbarium.



डॉ. एम. यु. शरीफ़/DR. M. U. SHARIEF
वैज्ञानिक 'एफ' एवं कार्यालयाध्यक्ष/
SCIENTIST 'F' & HEAD OF OFFICE

सेवा में / To

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Ph.D. Research Scholar
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Avinashilingam Institute for Home Science &
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COIMBATORE – 641 043