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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 ashed. 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

Acid Detergent Fibre (ADF)

1g of powdered sample and 100 ml 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, even level. The container was removed, swirled and filtered the contents through a preweighed 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^oC for overnight and 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 fiber and S is the weight of the sample.

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^oC 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 550 ^oC for about 3 h. 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

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

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 form 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, allowed to stand for 1 hr. 1 ml of the solution was taken and diluted to 100 ml. 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
(Walkey 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 determined 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 and 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_2O7	= 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 Yml of FeSO4 reduces Y/X* 10ml	
Hence actual quantity of 1N K2Cr2O7 used for	
oxidation of organic matter	= 10 - (10* Y/X)
1ml of 1N K2Cr2O7	= 0.003gm of ‘C’
Therefore 10 – (10*Y/X) ml of 1N K2Cr2O7	= 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.

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

Where,

T.V = Titre value.

0.00028 = 1ml of 0.02 N sulphuric acid utilized.

10 = Volume of extract taken for distillation (ml).

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,Conc. ammonium hydroxide,Conc. nitric acid,Solid ammonium nitrate, Ammonium molybdate solution – 20 %, Potassium hydroxide – 0.1619N, Nitric acid - 0.1619N and 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 a 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, 0.1619N 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 0.1619N 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

Therefore, actual volume of 0.1619N KOH used to dissolve the precipitate	= (a-b)
1 ml of 0.1619N KOH	= 0.0005gm P_2O_5
(a-b) ml of 0.1619N KOH	= 0.0005 x (a-b) x gm P_2O_5

This was present in 200 ml of HCL extract

Therfore, 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 x100/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 100 ppm.

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 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 Erichrome 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 and Erichrome Black – T indicator.

Procedure

Calcium alone

Pipette out 10 ml of seaqui oxide filterate 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 seaqui oxide 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 Erichrome 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 R2O3filterate made upto	= 250 ml
Volume of R2O3filterate 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 formagnesium 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

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

Percentage of magnesium on moisture free basis

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

M = Moisture basis

Appendix - 8

ESTIMATION OF PROTEIN

(Lowry *et al*., 1951)

Principle

The blue colour developed by the reduction of the phosphomolybdic-phosphotungstic 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 50 mg 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 10 minutes. 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

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: 200 mg anthrone was dissolved in 100ml of ice cold 95% H2SO4 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

100 mg 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

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 amounts of chlorophyll contents were calculated.

Materials

Analytical grade acetone was diluted to 80 % acetone (prechilled)

Procedure

Accurately weighed 1g of finely cut and well mixed representative leaf sample. It was ground to a fine pulp with the addition of 20 ml 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 20 ml 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 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.

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

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

(iii) Total chlorophyll mg g^{-1} 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

ESTIMATION OF LEGHAEMOGLOBIN

(Appleby and Bergersen, 1980)

Principle

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

Reagents

- Diluent buffer: 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 or thawed nodules were mixed with 1-3 volumes of phosphate buffer and macerated in a mixer. It was filtered through two layers of cheesecloth. The nodule 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 of alkaline pyridine reagent was added and mixed well. The solution becomes greenish-yellow due to the formation of ferric hemochrome.

The hemochrome was taken in equal quantity in two tubes. To one portion, few crystals of sodium 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 -12
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 (%) =

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

Appendix -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% KMnO4 and 100 ml of 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 taken	= 20g
Volume of N/50 H_2SO_4	= X ml (titre value)
1 ml of N/10 H_2SO_4	= 0.0014 g N
Therfore 1 ml of N/50 H_2SO_4	= 0.00028 gm N
X ml of N/50 H_2SO_4	= 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

AVAILABLE PHOSPHORUS IN SOIL

(BRAY 1 ,COLORIMETRY METHOD)

(Jackson, 1973)

Principle

The combination of HCL and NH4F 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 is estimated colorimetrically as available phosphorus.

Reagents

- NH4F solution (1N): 37g of NH4F 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 NH4F and 0.02 N HCL]: 15 ml of 1N NH4F and 25 ml of 0.5N HCL are mixed and the volume was up to 500 ml with distilled water and 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 filtered 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	= 5g
Volume of $NaHCO_3$	= 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
	= $X/10^6$ gm/ml
Therfore in 25 ml of solution	= $X/10^6*25$g

This is present in 50 ml of the extractant solution and 5 g 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 NH4+ 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. Filterates 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*25g
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

ANTIBACTERIAL ACTIVITY

KIRBY-BAUER METHOD

(Bauer *et al.*, 1996)

Procedure

Inoculum Preparation

The growth method is performed as follows:

At least three to five well-isolated colonies of the same morphological type are selected from an agar plate culture. The top of each colony is touched with a loop, and the growth is transferred into a tube containing 4 to 5 ml of a suitable broth medium, such as Mueller-Hinton broth. The broth culture is incubated at 35°C until it achieves or exceeds the turbidity (2 to 6 hours). The turbidity of the actively growing broth culture is adjusted with sterile saline or broth to obtain turbidity. This results in a suspension containing approximately 1 to 2 x 10^8 CFU/ml for *Escherichia coli* (gram negative) and *Staphylococccus aureus* (gram positive*).*

Inoculation of Test Plates

- Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab is dipped into the adjusted suspension. The swab should be rotated several times and pressed firmly on the inside wall of the tube above the fluid level. This will remove excess inoculum from the swab.
- The dried surface of a Mueller-Hinton agar plate is inoculated by streaking the swab over the entire sterile agar surface. This procedure is repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of

inoculum. As a final step, the rim of the agar is swabbed. Before applying the drug-impregnated discs, the lid may be kept open for 3 to 5 minutes, but no longer than 15 minutes, to allow any excess surface moisture to be absorbed.

- The media was punctured by making a well of 6 mm in diameter and filled with 50 µl of a sample. Further, the petri plates were placed inversely for complete diffusion and inhibition zones were examined by measuring the diameter (mm) formed around the well after 24 hrs incubation at 37°C. The zones were measured by using standard (Hi-Media) scale.

Appendix -17

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: DPPH - 2, 2-diphenyl-2-picryl hydrazyl hydrate (0.3mM in methanol) and 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 -18

HYDROGEN PEROXIDE SCAVENGING ACTIVITY

(Ruch *et al.*, 1989)

Principle

The UV absorption of hydrogen peroxide can be easily measured at 230 nm. On scavenging of hydrogen peroxide by the plant extract, the absorption decrease at this wavelength. This property is utilized to quantify their H_2O_2 scavenging ability.

Reagents: Phosphate buffer (0.1M, pH 7.4) and H_2O_2 (40mM) in phosphate buffer**.**

Procedure

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

$$\% \text{ scavenging of hydrogen peroxide} = \frac{(A_0 - A_1) \times 100}{A0}$$

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

Appendix -19

NITRIC OXIDE RADICAL SCAVENGING ACTIVITY

(Green *et al.*, 1982)

Principle

At physiological pH, sodium nitroprusside generate nitric oxide which interacts with O2 to produce nitrite ions, which is measured at 546 nm.

Reagent: Sodium nitroprusside (100 mM)**,**pH buffers saline (PBS) pH 7.4 and 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 -20

REDUCING POWER ASSAY

(Oyaizu, 1986)

Principle

Substances, which have reduction potential, react with potassium ferricyanide (Fe3+) to form potassium ferrocyanide (Fe2+), which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700 nm.

Potassium ferricyanide + Ferri c chloride ⟶ Potassium ferrocyanide + Ferrous chloride

Reagents: Potassium ferricyanide (1%), Phosphate buffer (0.2 M, pH 6.6), Trichloro acetic acid (10%), Ferric chloride (0.1%) and 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% trichloroacetic 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 -21

PRELIMINARY PHYTOCHEMICAL SCREENING

(Harborne, 1984)

Test for Alkaloids

Mayer's test: To 1ml of extract, 2ml of Conc. HCl was added. Then, a few drops of Mayer's reagent were added. Green colour or white precipitate indicates the presence of alkaloids.

Test for Anthraquinones

Borntrager's Test: To 1g of plant powder, 5 to 10 ml of dilute sulphuric acid is added and is boiled and filtered. The filtrate is treated with chloroform or benzene and then dilute nitric acid is added. Appearance of pink to red colour indicates the presence of anthraquinones.

Test for Amino acids

To 1ml of filterate, few drops of 0.2% ninhydrin was added and heated for 5 minutes. Formation of blue colour indicated the presence of aminoacid.

Test for Flavonoids

To 1ml of extract, 1ml of neutral ferric chloride was added. The formation of brown colour confirmed the presence of flavonoids

Test for Glycosides

Baljet test : Extract when mixed with picric acid gives orange color which is indicative of the presence of glycosides.

Test for Phenol

To 1ml of extract 5ml of Folin ciocalteau reagent and 4ml of sodium carbonate was added. Appearance of blue colour showed the presence of phenol**.**

Test for Steroids

To 1ml of the filterate, 10ml of Chloroform and 10ml of sulphuric acid was added slowly by the sides of the test tube. Upper layer turning red and the sulphuric acid layer turning yellow with green fluorescent indicates the presence of steroids.

Test for Saponins

Foaming test: When 1 ml of extract is shaken with water, formation of foam indicates presence of saponins

Test for Tannins

To 1ml of extract, 2ml of 0.1% Ferric chloride was added. Brownish green or blue black colouration indicates the presence of tannins.

Test for Terpenoids

To 1ml of filterate, 2ml of chloroform was added and few drops of concentrated sulphuric acid were added carefully. An interface with a reddish-brown colouration is formed showing the presence of terpenoids.

Appendix-22

MINERAL ANALYSIS

(Subbarao, 2003)

Reagents

Freshly prepared Tri-acid mixture of concentrated Nitric acid, Perchloric acid and concentrated Sulphuric acid in the ratio of 3:2:1 was used for digesting the samples.

Procedure

Weighed one gram of dried powdered seed sample and digested with 25 ml of tri-acid mixture in a 100 ml conical flask, shaken well so that no dry lumps were left. A clean and washed glass bead was dropped into flask to avoid bumping during digestion. The flask containing sample was allowed to stand for 3-4 hrs in a fume cupboard. Then this was heated on digestion heater and was watched out for fuming for the first hour. In case of excessive foaming which tends to overflow, the bulb of flask was immersed in cold water till digestion was completed. Then it was allowed to cool and the digested samples were filtered through Whatman filter paper No. 40 into a 100 ml volumetric flask. It was made sure that contents of digestion flask were quantatively transferred by rinsing the flask 3-4 times with deionized water.

The silica residue was washed on filter paper with dilute HCl (1:9) in order to wash down salt completely. The volume of collected filtrate was made 100 ml, this mineral solution was transferred in pre-acid washed polythene bottles and stored in cool place till use.The elements potassium, sodium, calcium, iron, zinc magnesium and manganese were measured in the solutions prepared from Tri-acid digested samples.

A blank was run along with each set of samples. Sodium and calcium were estimated with the help of Flame photometer, whereas Atomic Absorption Spectrophotometer (AAS) 4129 was used for the estimation of Potassium, iron, magnesium and zinc at different wavelengths of 324 nm, 248 nm, 213 nm and 279 nm respectively. Dilutions, wherever necessary, were made with the help of double distilled water and dilution factor was incorporated in the final calculations if needed.

Calculations

The amounts of sodium and calcium were calculated from the reading of Flame photometer (F) with the help of following equation:

$$\text{Concentrated of element (mg/100g)} = \frac{F \times V \times df}{10 \times W}$$

Where,

F = Observed reading of flame photometer

V = Volume of the undiluted sample

W = Sample weight in g

df = Dilution factor

The concentrations of the potassium, iron, magnesium and zinc in ppm were digitally displayed. The ppm value for each element was divided by 10 for conversion into mg/100g.

Appendix - 23

ESTIMATION OF PHOSPHORUS
NITRO-VANADO-MOLYBDATE METHOD
(Jackson, 1958)

Nitro-vanado-molybdate method (Jackson, 1958) was used for spectro-photometrically determining the phosphorus content of the test crop leaves. Orthophosphates form a yellow coloured phosho-vanado-molybdate complex in presence of V5+ and Mo_6+, which shows an optimal absorption at wavelength 440nm. 5ml aliquote from the prepared sample was mixed with 5ml of nitro-vanado-molybdate reagent (100ml of 5% ammonium molybdate solution + 100ml of 0.25% ammonium vanadate solution + 100ml of diluted nitric acid). The volume was made up to 25ml with distilled water. The intensity of the yellow color obtained was measured at 440nm using spectro-photometer (Cary-60, Agilent Technologies). Phosphorus content was expressed in % and calculated using the below-mentioned formula.

$$\text{Phosphorous (\%)} = \frac{\text{Biuret reading} \times \text{Dilution factor} \times \text{Graph factor}}{10000}$$

Annexure-1

Plant Authentication Certificate

Vigna unguiculata (L.) Walp.

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GOVERNMENT OF INDIA
पर्यावरण,वनऔरजलवायुपरिवर्तनमंत्रालय
MINISTRY OF ENVIRONMENT, FOREST & CLIMATE CHANGE
भारतीयवनस्पतिसर्वेक्षण
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bsisc@rediffmail.com

सं. भा.व.स./द.क्षे.के./No.: BSI/SRC/5/23/2022/Tech /395 दिनांक/Date: 18th August 2022

पादप प्रमाणीकरण प्रमाणपत्र / PLANT AUTHENTICATION CERTIFICATE

The plant specimen given by you for authentication is identified as ***Vigna unguiculata*** **(L.) Walp.- 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.

मु. उ. शरीफ 18/08/2022

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

सेवा में / To

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

Annexure-2

Plant Authentication Certificate

Vigna unguiculata subsp *sesquipedalis* (L.) Verdc.

भारतसरकार
GOVERNMENT OF INDIA
पर्यावरण, वन और जलवायु परिवर्तन मंत्रालय
MINISTRY OF ENVIRONMENT, FOREST & CLIMATE CHANGE
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BOTANICAL SURVEY OF INDIA

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सं. भा.व.स./द.क्षे.के./No.: BSI/SRC/5/23/2022/Tech. / 188

दिनांक/Date: 9th June 2022

पौधे प्रमाणीकरण प्रमाणपत्र / PLANT AUTHENTICATION CERTIFICATE

The plant specimen brought by you for authentication is identified as *Vigna unguiculata* subsp. *sesquipedalis* (L.) Verdc. - FABACEAE. The identified specimen is returned herewith for preservation in their College/ Department/ Institution Herbarium.

09/06/2022

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

सेवा में / To

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

Annexure -3

Plant Authentication Certificate

***Clitoria ternatea* L**

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पर्यावरण, वन और जलवायु परिवर्तन मंत्रालय
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सं. भा.व.स./द.क्षे.के./No.: BSI/SRC/5/23/2022/Tech. | 186

दिनांक/Date: 9th June 2022

पौधे प्रमाणीकरण प्रमाणपत्र / PLANT AUTHENTICATION CERTIFICATE

The plant specimen brought by you for authentication is identified as *Clitoria ternatea* L. - 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

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

Annexure - 4

Plant Authentication Certificate

Amaranthus tricolor L.

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GOVERNMENT OF INDIA
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पौधे प्रमाणीकरण प्रमाणपत्र / PLANT AUTHENTICATION CERTIFICATE

The plant specimen brought by you for authentication is identified as *Amaranthus tricolor* L. - AMARANTHACEAE. The identified specimen is returned herewith for preservation in their College/ Department/ Institution Herbarium.

09/06/2022

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

सेवा में / To

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