Appendices

APPENDICES

APPENDIX – I
ESTIMATION OF CHLOROPHYLL
(Witham *et al.*, 1971)

Principle

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

Reagents

80% Acetone

Procedure

Weighed 1g of finely cut and well mixed representative sample of leaf or fruit tissue into a clean mortar. Ground the tissue to a fine pulp with the addition of 20 ml of 80% acetone. Centrifuge (5000 rpm for 5 minutes) and transferred the supernatant to a 100ml volumetric flask. Ground the residue with 20 ml of 80% acetone, centrifuged and transferred the supernatant to the same volumetric flask. Repeated this procedure until the residue was colourless. Washed the mortar and pestle thoroughly with 80% acetone and collected the clear washings in the volumetric flask. Made up the volume to 100 ml with 80% acetone. Read the absorbance of the solution at 645, 663 and 652 nm against the solvent (80% acetone) blank.

Calculation

Calculated the amount of chlorophyll present in the extract mg chlorophyll per g tissue using the following equations

$$\text{mg chlorophyll a/g tissue} = 12.7(A_{663}) - 2.69(A_{645}) \times \frac{V}{1000 \times W}$$

$$\text{mg chlorophyll b/g tissue} = 22.9(A_{645}) - 4.68(A_{663}) \times \frac{V}{1000 \times W}$$

$$\text{mg total chlorophyll/g tissue} = 20.2(A_{645}) + 8.02(A_{663}) \times \frac{V}{1000 \times W}$$

Where,

A- Absorbance at specific wavelengths,

V - Final volume of chlorophyll extract in 80% acetone

W - Fresh weight of tissue extract

APPENDIX – II
ESTIMATION OF TOTAL CAROTENOID
(Goodwin, 1954)

Principle

Carotenoids was extracted in 80% acetone and absorption at 473 nm were read in a spectrophotometry.

Reagents

80% Acetone

Procedure

Weighed 1gm of fresh leaves, cut into small pieces and homogenised in a mortar and pestle with excess acetone. Decanted and filtered the supernatant and added the sufficient quantity of 80% acetone and repeated the extraction. Transferred the contents from the mortar and washed with acetone until it became colourless. Pooled the filtrates and made up the volume to 100ml in a volumetric flask. Transferred 50ml of extract in to a 50ml of volumetric flask and diluted by making up the volume with 80% acetone. Measured the absorbance at 473nm using a extinction coefficient (E_1 em of 2500 as a average value). The values were expressed as mg carotenoids per gram fresh weight.

Calculation

$$\text{Carotenoids (mg/g fresh weight)} = \frac{OD_{645} \times \text{Volume} \times 1000}{2500 \times 100 \times 1 \times \text{Weight}}$$

E_1 em of carotenoids dissolved in 100ml of solvent in 1 cm light path,

Where 1cm = path of light

Volume = Volume of extract in ml.

APPENDIX - III
ESTIMATION OF PROTEIN
(Lowry *et al.*, 1951)

Principle

The blue colour was developed by the reduction of the phosphomolybdic phosphotungstic components. 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 copper tartarate were measured in the Lowry's method.

Reagents

1) Reagent A: 2% sodium carbonate in 0.1N sodium hydroxide.

2) Reagent B: 0.5% copper sulphate ($CuSo_4.5H_2O$) in 1% potassium sodium tartarate.

3) Reagent C: Alkaline copper sulphate solution: Mixed 50ml of A and 1ml of B prior to use.

4) Reagent D: Folin-Ciocalteau Reagent - Refluxed gently for 10 hours a mixture consisting of 100g sodium tungstate, 25gm sodium molybdate, 700ml water, 50ml of 85% phosphoric acid and 100ml of concentrated hydrochloric acid in a 1.5 litre flask. Added 150gm of lithium sulphate, 50ml water and a few drops of bromine water. Boiled the mixture for 15min without condenser to remove excess bromine. Cooled, diluted to 1 litre and filtered.

5) Stock standard protein solution: Weighed accurately 50mg of bovine serum albumin and dissolved in 0.1N NaOH and made up to 50ml in a standard flask.

6) Working standard: Diluted 10ml of the stock solution to 50ml with distilled water in a standard flask. 1ml of the solution contains 200 μg of proteins.

Procedure
Extraction of protein from sample

Weighed 500mg of the sample and ground well with a pestle and mortar in 5-10ml of the phosphate buffer. Centrifuged and used the supernatant for the protein estimation.

Estimation of protein

Pipetted out 0.2, 0.4, 0.6, 0.8 and 1ml of the working standard into a series of test tubes. Pipetted out 0.1ml of the sample extract in another test tube. The volume was made up to 1ml in all the test tubes. A tube with 1ml of water served as a blank. Added 5ml of reagent C to all the test tubes including the blank. Mixed well and allowed to stand for 10minutes and then 0.5ml of reagent D was added, mixed well and incubated at room temperature in the dark for 30minutes. Blue colour was developed. Readings were taken in spectrophotometer at 660nm. Standard graph was drawn and the amount of protein in the sample was calculated.

APPENDIX - IV
ESTIMATION OF TOTAL CARBOHYDRATE
(Hedge and Hofreiter, 1962)

Principle

Carbohydrates were first hydrolysed into simple sugar using dilute hydrochloric acid. In hot acidic medium glucose is dehydrated to hydroxyl methyl furfural. This compound forms with anthrone a green coloured product with an absorption maximum at 630nm.

Reagents

1) 2.5N hydrochloric acid

2) 95% sulphuric acid

3) **Anthrone reagent:** Dissolved 200mg of anthrone in 100ml of ice cold 95% sulphuric acid. Prepared fresh before use.

4) **Stock standard glucose solution:** 100mg of glucose was dissolved in 100ml of distilled water.

5) **Working standard:** 10ml of stock solution was diluted to 100ml with distilled water. Stored in a refrigerator after adding a few drops of toluene.

Procedure
Extraction of carbohydrates

Weighed 100mg of the sample into a boiling tube. Hydrolysed by keeping it in a boiling water bath for 3 hours with 5ml of 2.5 N hydrochloric acid and cooled to room temperature. They were neutralized it with solid sodium carbonate until the

effervescence ceases. Made up the volume to 100ml and centrifuged, collected the supernantant and took 0.5 to 1 ml aliquots for analysis.

Estimation of total carbohydrate

Prepared the standards by taking 0, 0.2, 0.4, 0.6, 0.8 and 1ml of working standard. 'O' served as blank. Made up the volume to 1 ml in all the tubes including the sample tubes by adding distilled water. Then added 4 ml of anthrone reagent. Heated for 8 minutes in a boiling water bath. Cooled rapidly and read the green to dark green colour at 630 nm. Draw a standard graph by plotting concentration of standard on the X- axis versus absorbance on the Y-axis. From the graph calculated the amount of carbohydrates present in the sample tube.

APPENDIX - V
ESTIMATION OF TOTAL PHENOL
(Bray and Thorpe, 1954)

Principle

Estimation of phenol with folin-phenol reagent was based on the reaction between phenol and an oxidizing agent phosphomolybdate which results in the formation of a blue colour. The intensity of colour developed was read colorimetrically at 650nm.

Reagents

1) **Folin-phenol reagent:** Dissolved 100gm of sodium tungstate and 25gm of sodium molybdate in 700ml of water. Added 50ml of orthophosphate and 100ml of concentrated hydrochloric acid boiled under reflux gently for 10 hours. Cooled and added 150gm lithium sulphate, dissolved in 50ml water and 4-5 drops of liquid bromine. Boiled the mixture without condenser for 15minutes to remove the excess bromine. Cooled, diluted to 1 litre with distilled water and then filtered. Just before use 1 volume of the stock solution is diluted with 2 volume of water.

2) **Sodium carbonate:** 20%

3) **Catechol stock standard:** 100mg of catechol was dissolved in 100ml of distilled water.

4) Working standard was prepared by diluting 10ml of stock to 100ml.

Procedure

1ml of the extract was pipetted out into a test tube and 1ml of folin-phenol reagent followed by 2ml of sodium carbonate was added. The tubes was shaken and heated in a boiling water bath for exactly 1 minute and then it was cooled under the tap water. The blue colour developed was diluted to 25ml with distilled water and its absorbance was measured colorimetrically at 650nm. The unknown was read from a standard curve made from different concentrations of catechol. A blank containing all the reagent minus plant extract was used to adjust the absorbance to zero.

APPENDIX - VI
ESTIMATION OF NITROGEN
MICROKJELDAHL METHOD
(Humphries, 1956)

Principle

Total nitrogen was the sum of ammonium nitrogen and organic nitrogen. The nitrogen was converted to ammonium sulphate when treated with the sulphuric acid. An excess of alkali were added to liberate ammonia and distilled. This solution was titrated with sulphuric acid after absorption in boric acid solution.

Reagent

1) **Diacid:** Concentrated sulphuric acid and perchloric acid were mixed in the ratio 1:1.
2) **Mixed indicatior**: Dissolved 0.5 gm of bromocresol green and 0.1 gm of methyl red in 100ml of ethyl alcohol.
3) **Boric acid**: 3%
4) **Sodium hydroxide**: 40%
5) **Sulphuric acid:** 1/70 N

Procedure

Ground the dried sample and made it to a fine powder. Took 0.5 gm of sample in a microkjeldahl flask and added 12ml of diacid. Digested the sample over a heated sand bath. Made up the volume to 100 ml with distilled water.

Pipetted out 10ml aliquot into a microkjeldahl distillation apparatus. Kept at the delivery end, 10 ml of 2% boric acid mixed with indicator in a 100ml conical flask. Added 10ml of 40% sodium hydroxide into distillation apparatus and steamed the distillation until a blue colour was reached. The distillate was titrated against N/70 sulphuric acid until the red colour was appeared.

Calculation

$$\text{Nitrogen Content (1\%)} = \frac{0.0028 \times \text{Titrevalue} \times 100}{10} \times \frac{100}{0.5}$$

APPENDIX - VII
ESTIMATION OF PHOSPHORUS
(Raguramulu *et al.*, 2003)

Principle

Acid molybdate reagents react with inorganic phosphate to form phosphomolybdic acid. The hexavalent molybdenum of phosphomolybdic acid is reduced to give a blue compound, which is estimated colorimetrically.

Reagents

1. **Molybdate I solution :** 2.5% ammonium molybdate in 5N sulphuric acid.
2. **Amino Naphthol Sulphonic Acid (ANSA):** Added 0.5g of 1, 2, 4 – amino naphthol sulphonic acid in 195ml of 15% sodium bi sulphite and added 5 ml of 20% sodium sulphite and shaken until it is dissolved.
3. **Stock standard phosphate solution:** 35.1mg of potassium dihydrogen phosphate was dissolved in 20ml of water. 1.0 ml of 10N sulphuric acid was added and volume was made up to 100ml with water.
4. **Working standard:** 10 ml of stock standard phosphate solution was made upto 100ml with distilled water.

Procedure

2g of vegetable samples was taken and were ashed. Dissolved in 2 to 3 drops of concentrated hydrochloric acid and made up to 25ml with distilled water. Into a series of test tubes pipetted out 0.5, 1.0, 1.5, 2.0 and 2.5ml of working standard

solution and made up the volume to 4.7ml with water and added 0.5 ml of molybdate I solution and 0.2 ml of ANSA. 0.1 ml of the ash solution was taken and treated similarly. The colour developed was read after 20 minutes in a colorimeter using red filter against a reagent blank.

APPENDIX - VIII
ESTIMATION OF POTASSIUM
(Jackson, 1975)

Principle

In flame photometry, the solution under test is passed under careful conditions as a very fine spray in the air supply to a burner. The flame, the solution evaporates and the salt dissociates to give a neutral atoms. A very small proportion of this move into a higher energy state. When this excited atoms fall back to the ground state the light emitted of characteristic wave length was measured. Potassium was estimated at 710nm.

Reagents

1. Triple acid mixture: Concentrated nitric acid, perchloric acid and sulphuric acid were mixed in the ratio of 3:2:1.

Procedure

Sample preparation was done as for phosphorus. 5ml of the acid extract was pipetted out into a vial and the transmission was read in the flame photometer. Using a potassium standard curve, the concentration to potassium was calculated

Potassium standard curve

1.907gm of analar grade potassium chloride was dissolved in 100ml of distilled water to get 1000ppm of potassium. 100ml of 1000ppm of potassium was diluted to 1l to get 100ppm of potassium. From this a series of potassium standards ranging from 0-100ppm were prepared and the percentage transmission was read on a flame photometer to construct a standard curve.

Calculation

$$\text{Potassium content (\%)} = \frac{\text{Potassium concentration in ppm}}{10^6} \times \frac{100}{5} \times \frac{100}{5}$$

APPENDIX-IX
ESTIMATION OF ORGANIC CARBON
(Jackson, 1973)

Principle

Organic matter in the soil was oxidised with a mixture of potassium dichromate and concentrated sulphuric acid utilising the heat of solution of sulphuric acid. Unused potassium dichromate was back – titrated with ferrous ammonium sulphate.

Reagents

1) Standard potassium dichromate solution: Dissolved exactly 49.04 gm potassium dichromate in distilled water and diluted to 1 litre in volumetric flask.

2) Ferrous ammonium sulphate: Dissolved 196.1 gm of ferrous ammonium sulphate in about 800ml of water. Added 20 ml of concentrated sulphuric acid. Cooled and diluted to 1 litre in volumetric flask.

3) Diphenyl amine indicator: Dissolved 0.5 gm of diphenylamine in a mixture of 20ml of water and 100ml of concentrated sulphuric acid.

4) Sulphuric acid not less than 96% concentration.

5) Orthophosphoric acid – 85%

Procedure

Accurately weighed 1 gm of the sample. Two blanks were included to standardize ferrous ammonium sulphate. Added exactly 10 ml of dichromate solution. The flasks were swirled gently and kept on asbestos sheet. Rapidly added 20 ml of concentrated sulphuric acid by directing steam into the suspension. The flasks were swirled again two to three times. Allowed the flasks to stand on asbestos sheet for 30 minutes added about 200ml of distilled water. After addition of 10 ml of phosphoric acid and 1ml of diphenylamine indicator titrated the contents with ferrous ammonium sulphate till the colour turned from blue violet to green.

Calculation

$$\text{Organic carbon (\%)} = \frac{10\,(B-T)}{B} \times \frac{0.003 \times 100}{\text{Weight of sample (gm)}}$$

Where

B = Volume of ferrous ammonium sulphate required for blank titration.

T = Volume of ferrous ammonium sulphate required for titration of sample.

APPENDIX - X
ESTIMATION OF CALCIUM AND MAGNESIUM
VERSENATE METHOD
(Cheng and Bray, 1951)

Reagents

1) **Ammonium chloride-ammonium hydroxide buffer solution:** Dissolved 67.5 gm of ammonium chloride in 570 ml of concentrated ammonium hydroxide and made up to one litre.

2) **Eriochrome –Black –T-indicator:** Dissolved 1 gm of Eriochrome - Black - T in 100ml of triethanolamine.

3) **Sodium hydroxide:** 10%

4) **Murexide indicator**: Thoroughly mixed 0.5gm of ammonium purpurate with 100 gm of potassium sulphate.

Procedure

a) Calcium: to 10 ml of the aliquot in a porcelain disk, added 10 ml of 10% sodium hydroxide solution and a pinch (0.3gm) of murexide indicator. Titrated against 0.02N ethylene diamine tetra acetate till the pink solution turned to a characteristic violet colour. The titrate represented the value for calcium alone.

b) Calcium + Magnesium: To 10 ml of the aliquot added 5ml of water, 15ml of ammonium chloride- ammonium hydroxide buffer and a few drops of Eriochrome – Black –T indicator. Titrated the dark purple coloured solution against 0.02N ethylene diamine tetracetate till the colour turned to blue. The titre value represented the value for both calcium and magnesium.

APPENDIX - XI
SOIL ACIDITY
(Shoemaker, 1961)

The most important of all soil tests is perhaps the soil pH. It is essential for the interpretation of the other soil test results, since the availability of P, Ca, Mg, Fe, Mn,

Zn, Cu and B are greatly influenced by the soil pH. An acid soil test, coupled with the knowledge for the soil type and the crops to be grown, permits a recommendation on the quantity of ground limestone needed to bring the soil pH to a desired level.

Usually in soil testing laboratories, the soil pH is measured in 1: 2.5, soil : water suspension using a glass electrode pH meter. When the measured soil pH is 6.4 and above there is no need to apply limestone. Ground limestone is recommended when the soil pH is 6.3 and below. For such soils, the lime requirement can be determined by the procedure given below.

Reagents required

1. Extractant buffer

Dissolve 1.8g of paranitrophenol, 2.0 g of calcium acetate, 2.5ml of triethanolamine, 3g of potassium chromate and 53.1 g of calcium chloride in 1l of distilled water and adjusting the pH to 7.5 using dilute sodium hydroxide.

Procedure

Weigh 5g of the given soil and transfer to a 100ml beaker. Add 5ml of distilled water and 10ml of the extractant buffer. Shake the contents of the beaker for 10 minutes continuously and measure the pH of the soil buffer suspension. Calculate the lime requirement.

SOIL SALINITY

When a soil contains an excess amount of soluble salts, it is termed as saline soil. Soluble salt analysis for soils generally concerns whether enough salt is present to cause interference with normal intake of water by the crop plants. In saline soils, though enough amount of water is kept in enough storage in soil profile, the crop growth is restricted because of the greater soil water potential than in the normal soil, which the plant must overcome to withdraw salt free water. A fairly quantitative estimate of salt content of solutions extracted from soils can be made from their electrical conductance. As the amount of salts in the solution increases, the electrical conductivity also increases. The electrical conductivity is usually measured in terms of resistance offered to the flow of current using a conductivity bridge as given below (Jackson, 1973).

Reagents required

1. Saturated solution of calcium sulphate
2. 0.01N potassium chloride

Procedure

Weigh 20gm of soil and transfer to a 100ml beaker and add 50ml of distilled water. Stir this 1: 2.5, soil: water suspension well and allow it to stand for half an hour. Switch on the conductivity bridge. Check the instrument with saturated calcium sulphate and 0.01 N potassium chloride (the electrical conductivity of these two solutions are 2.2.dSm^{-1} and 1.41dSm^{-1} respectively) before proceeding with the measurement on the sample. Wash the electrode with distilled water. Immerse it into soil suspension or suck the supernatant solution into the electrode bulb if the electrode is of pipette type. Rotate the meter knob until the magic eye of the null indicator is at its widest. The readings of the scale at this position indicate the electrical conductivity. Multiply this reading by the cell constant, which is noted on the electrode itself to get the salinity scale, which is given below for interpretation.

Interpretation based on EC measurements

EC (dSm^{-1})	Salinity effect	Interpretation
0.0 -1.0	Harmless	Salinity
1.1 - 3.0	Critical	Limit yield of sensitive crops
3.1 and above	Injurious	Limit yield of many crops