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 and 663 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 CAROTENOIDS
(Zakaria *et al.*, 1979)

Principle

The total carotenoids in the sample were extracted in petroleum ether. The total carotenoids were estimated in a UV/Visible spectrophotometer at 450nm.

Reagents

1. Petrolum ether (40°-60°)
2. Anhydrous sodium sulphate
3. Calcium carbonate
4. 12% alcoholic potassium hydroxide

Procedure

Weighed 5-10g of the sample. Saponified for about 30 minutes in a shaking water bath at 37°C after extracting the sample in 12%alcoholic KOH. Transferred the saponified extract into a separating funnel packed with glass wool and $CaCO_3$ containing 10 to 15ml of petroleum ether layer. Transferred the lower aqueous phase to another separating funnel, and the petroleum ether extract containing the carotenoid pigments to amber coloured bottle. Repeated the extraction of the aqueous phase. To the petroleum ether extract added a small quantity of anhydrous Na_2SO_4 to remove the turbidity. Noted the final volume of the petroleum ether extract and diluted if needed by a known dilution factor. The absorbance of the extract at 450nm and 503nm was noted in a spectrophotometer.

$$\text{Amount of total carotenoids present} = \frac{P \times 4 \times V \times 100}{W}$$

P = Optical Density of the sample

V = Volume of the sample

W = Weight of the sample

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 PHENOL
(Malick and Singh, 1980)

Principle

Phenols react with phosphomolybdic acid in Folin - ciocalteau reagent in alkaline medium and produce blue coloured complex (molybdenum blue), which is read in a spectrometer at 650nm.

Reagents

1. 80%ethanol
2. Diluted Folin - Ciocalteau reagent
3. 20% sodium carbonate
4. **Stock solution**: Dissolved 100mg of catechol is made up with 100ml distilled water
5. **Working standard**: 10ml of stock standard was diluted to 100ml. 1.0ml of this contains 100µg of catechol.

Procedure

1g of sample was homogenized using 20ml of 80% ethanol. The homogenate was centrifuged at 10,000rpm for 20 minutes. The supernatant was saved. The residue was reextracted with 10ml of 80% ethanol, centrifuged and collected the supernatant and evaporated to dryness. The residue was dissolved in a known volume of distilled water (50ml) and 2.0ml was taken for the experiment. A working standard of 0.5 – 2.5ml catechol solution corresponding to 50 - 250µg of catechol were pipetted out into a series of test tubes. The volume was made upto 2.5ml with water. To all the tubes added 0.5ml of diluted Folin – Ciocalteau reagent. After 3 minutes, added 2.0ml of 20% Na_2CO_3 solution to each tube and mixed thoroughly. The tubes were placed in a boiling water bath for exactly one minute. Cooled and measured at 650nm against a reagent blank. Constructed a standard graph by plotting the concentration of catechol on X-axis and absorbance on Y-axis. From the graph, the amount of total phenol present in the sample was estimated and expressed as mg of total phenols per g of the sample.

APPENDIX V
ESTIMATION OF REDUCING SUGAR
(Somogyi, 1952).

Principle

The reducing sugars when heated with alkaline copper tartarate reduce the copper from the cupric to cuprous state and thus cuprous oxide is formed. When cuprous oxide is treated with arsenomolybdic acid, the reduction of molybdic acid to molybdenum blue takes place. The blue colour developed is compared with a set of standards in a colorimeter at 620nm.

Regents

1. **Alkaline copper tartarate**

A. dissolved 2.5g of anhydrous sodium carbonate, 2g of sodium bicarbonate, 2.5g of potassium sodium tartarte and 20g of anhydrous sodium sulphate in 80ml of water and made up to 100ml.

B. dissolved 15g of copper sulphate in a small volume of distilled water. Added one drop of sulphuric acid and made upto 100ml. before use, 4ml of B and 96ml of solution A were mixed.

2. **Arsenomolbdate reagent**: Dissolved 2.5g of ammonium molybdate in 45ml water. Added 2.5ml of sulphuric acid and mixed well. Then 0.3g of disodium hydrogen arsenate was dissolved in 25ml of water. Mixed well and incubated at 37°c for 24 to 48 hours.
3. **Standard glucose solution:** 100mg of glucose was dissolved in 100ml of distilled water.
4. **Working standard**: 10ml of stock was diluted to 100ml distilled water.

Procedure

Weighed 100mg of the sample and the sugars were extracted with hot 80% ethanol twice (5ml each time). The supernatant was collected and evaporated by keeping it on a water bath at 80°C. 10ml of water was added and the sugars were dissolved. 0.2ml of the extract was pipetted out in test tubes. 0.2, 0.4, 0.6, 0.8 and 1.0ml of working standard solutions were also pipetted out into a series of test tubes. The volume in all tubes were made up to 2ml with distilled water. Tube with 2ml of distilled water was kept as blank. 1ml of alkaline copper tartarate reagent was added to all the tubes. The tubes were kept in boilning water bath for 10 minutes. The tubes were cooled and 1ml of arsenomolybdic acid reagent was added to all the tubes. The volume in all the tubes were made up 10ml with water. The absorbance of blue colour was read at 620nm after 10minutes. From the graph drawn, the amount of reducing sugars present in the sample was calculated

APPENDIX VI
ESTIMATION OF PHOSPHORUS
(Fiske and subbarow's, Oser,1971)

Principle

Phosphorus reacts with molybdic acid to form phospho molybdic acid. On treatment with 1, 2, 4 amino napthol sulphonic acid (ANSA), phosphosmolybdic acid is reduced to produce a deep colour (molybdenum blue) which is a mixture of lower acids of molybdenum. The blue colour is measured spectrophotometrically at 660 nm.

Reagents

1. **25 % ammonium molybdate**: Dissolved 2.5g of ammonium molybdate in 200 ml water and transferred to 1L flask containing 300 ml of 10 N H_2SO_4 and diluted to 1L with water.

2. **Amino napthol sulphonic acid Reagent (ANSA):** 195 ml of 15% Sodium bisulphate is taken in a glass stoppered cylinder and 0.5 g of 1, 2, 4 amino sulphonic acid is added to it followed by 5 ml 0f 20% Sodium sulphite. It is stoppered and shaken until the powder is dissolved. If the solution is not complete, added more sodium sulphite, 1.0 ml at a time with shaking. The solution is then transferred to a brown glass bottle and stored in the cold. This solution is usable for 4 week.

3. **Stock standard phosphate solution**: Accurately weighed 35.1 mg of mono Potassium hydrogen phosphate (KH_2PO_4), dissolved in water and added 1.0 ml of 10 N sulphuric acid and made up to 100 ml with water. 1.0 ml of this solution contains 80 µg of phosphorus.

4. **Working standard solution:** 10 ml of the stock standard phosphate solution was diluted to 100 ml with distilled water. 1.0 ml of the working standard solution contains 8 µg of phosphorus.

Procedure

Into a series of test tubes pipetteed out 1.0 – 5.0 ml of working standard solution corresponding to µg values 8 – 40. 0.5 ml of the sample (ash) solution was taken in separate test tube. The volume in all the tubes was made up to 8.6 ml with distilled water. Set up a blank with 8.6 ml of distilled water. Added 1.0 ml of 2.5 % ammonium molybdate and 0.4 ml of ANSA to all the tubes. Mixed well and allowed to stand for 10 minutes. A standard graph was drawn by plotting the concentration of phosphorus on X – axis and the optical density on Y – axis. From this the concentration of phosphorus in the sample solution is calculated.

APPENDIX VII
ESTIMATION OF IRON
(Wong's, Oser, 1971)

Principle

Iron is determined colorimetrically with ferric iron which gives a blood red colour with potassium thiocyanate which is measured at 540nm.

Reagents

1. 30% H_2SO_4
2. 7% Potassium per sulphate solution
3. **40% Potassium thiocyanate solution**: 40 g KCNS is dissolved in 90 ml distilled water, 4.0 ml acetone is added and the volume is made up to 100 ml.
4. **Standard Iron solution**: 70.22 mg ferrous ammonium sulphate is dissolved in 10 ml distilled water and after of 0.5 ml of 1:1 HCL the solution is made up to 100 ml in standard flask (0.1 mg Fe/ml).
5. **Working standard**: Diluted 10 ml of stock standard solution to 100 ml. The concentration of working standard is 10µg Fe/ml.
6. **Sample preparation**: About 5.0 g of the sample is weighed accurately into a tared platinum (or porcelain) crucible (which has been previously heated to about 600°C and cooled). The crucible is placed over a low flame till all the material is completely charred followed by heating in a muffle furnace for about 3.5 hr at about 600°C. It is then cooled in a dessicator and weighed. To ensure completion of ashing, the crucible is again heated in the muffle furnace for 1 hr, cooled and weighed. This is repeated till two consecutive weight are the same and the ash is almost white or grayish white in colour.

Ash content (g/ 100g sample) = Wt. of the ash/ Wt. of the sample taken X 100

7. **Ash solution**: The ash is moistened with a small amount of distilled water (0.5 – 1.0 ml) and 5.0 ml of distilled HCL is added to it. The mixture is evaporated to dryness in a boiling water bath. Another 5.0 ml of HCL is added again and the solution is evaporated to dryness as before. 4.0 ml of HCL and a few ml of water are then added and the solution is warmed over a boiling water bath and filtered into a 50 ml volumetric flask using Whatmann No: 40 filter paper. After cooling, the volume is made up to 50 ml and suitable aliquots are used for the estimation of iron.

Procedure

Pipetted out 1.0 – 5.0 ml of working standard solution into the series of test tubes corresponding toμg values 10 – 50. 2.0 ml of the sample solution was taken in separate test tubes. The volume in all the tubes was made up to 6.5 ml with distilled water. Set up a blank with 6.5 ml of distilled water. Added 1.0 ml of 30% H_2SO_4, 1.0 ml of 7% Potassium per sulphate and 1.5 ml of 40 % Potassium thiocyanate solution were added. The red colour developed was measured within 20 min at 540nm in a spectrophotometer. A standard graph was drawn by plotting the concentration of iron on X- axis and the optical density on Y – axis. From this the concentration of iron in the sample solution was calculated.

APPENDIX VIII
ESTIMATION OF TOTAL FREE AMINO ACIDS
(Moore and Stein, 1948)

Principle

Ninhydrin, a powerful oxidising agent decarboxylates the alpha aminoacids and yields an intensly coloured bluish purple product which is colorimetrically measured at 570nm.

Regents

1. **Ninhydrin:** dissolved 0.8g of stannous chloride in 500ml of 0.2M citrate buffer. Added this solution to 20g of ninhydrin in 500ml of methyl cellosolve.
2. 0.2M citrate buffer
3. **Dilute solvent**: mixed equal volume of water and n- Propanol.
4. **Stock standard:** dissolved 50mg of leucine in 50ml of distilled water.
5. **Working standard:** diluted 10ml of stock standard to 100ml.

Procedure

Weighed 500mg of the plant sample and ground in mortar and pestle with small quantity of acid washed sand. To the homogenate added 5ml of 80% ethanol , centrifuged and collected the supernatant.

Estimation:

Pipetted 0.1ml to 1.0 ml of the standard solution into series of test tube and taken 0.1ml of extract and added 1.0ml of ninhydrin solution. Made up the volume to 2ml with distilled water. Heated the tubes in boiling water bath for 20minutes. Added 5ml of the diluent and mixed the contents and incubated for 15 minutes. The colour developed was read against a reagent blank in a colorimeter at 570nm. Reagent blank was prepared by taking 0.1ml of 80% ethanol

APPENDIX IX
ESTIMATION OF CATALASE ACTIVITY
(Luck, 1974)

The enzyme catalase has a double function and it catalyses the following

a. It decomposes hydrogen peroxide to give water and oxygen.

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

b. It oxidizes H^+ donors, for example Methanol, Formic acid, Phenol with the consumption of one mole of peroxide.

$$ROOH + AH_2 \xrightarrow{\text{Catalase}} H_2O + ROH + A$$

Principle

The UV light absorption of hydrogen peroxide solution can be easily measured between 230 and 250nm. On decomposition of hydrogen peroxide by catalase, the absorption decreases with time. The enzyme activity could be arrived at from this decrease. But this method is applicable only to enzyme solution, which do not absorb strongly at 230-250nm.

Reagents

1. **Phosphate buffer 0.067M (pH 7.0)** – Dissolve 3.522g of KH_2PO_4 and 7.268g of $K_2HPO_4.2H_2O$ in distilled water and made up the volume to one litre.
2. **Hydrogen peroxide – Phosphate buffer** – Dissolved 0.16 ml of H_2O_2 (10% W/V) to 100 ml phosphate buffer, prepared fresh. The absorbance of the solution should be about 0.5 at 240nm with 1 cm light path.

Procedure

Enzyme extract

The sample is homogenized in a prechilled mortar and pestle with M/150 phosphate buffer (assay buffer diluted 10 times) at 1 - 4°C and centrifuged. Stirred the sediment with cold phosphate buffer, allowed to stand in the cold with occasional shaking and then repeated the extraction once or twice. The extraction should not take more than 24 hr. The combined supernatants were used for the assay. Use fresh extracts for assay.

Assay

Read against a control cuvette 3ml of H_2O_2 containing the enzyme solution as in the phosphate buffer (M/15). Pipetted into the experimental cuvette 3ml of H_2O_2 phosphate buffer. Mixed in 0.01-0.04ml sample with the glass or plastic rod flattened at one end. Noted the time it required for a decrease in absorbance from 0.45-0.4. This value was used for calculations. If 't' was more than 60 seconds, repeated the measurement with more concentrated solution of the sample.

Calculation

Calculated the concentration of H_2O_2 using the extinction coefficient 0.036μ mole/ml.

APPENDIX X
ESTIMATION OF PEROXIDASE ACTIVITY
(Reddy *et al.*, 1995)

Principle

Peroxidase converts H_2O_2 to water and oxygen in the presence of hydrogen donor (pyrogallol or dianisidine) the oxidation of pyrogallol or dianisidine to colored product called purpurogalli is measured colorimetically.

Pyrogallol + H_2O_2 ⟶ Oxidized pyrogallol + H_2O

Reagents

1. Pyrogallol – 0.05M phosphate buffer (pH 6.5)
2. 1 % H_2O_2

Enzyme extract

Measured one gram of the sample with 5ml (w/v) 0.1M phosphate buffer (pH 6.5) in a homogenizer. Centrifuged the homogenate at 300g for 15 minutes. Used the supernatant as the enzyme source. All procedures were carried out at 0-5°C.

Procedure

Pipetted out 3ml of 0.05 M- pyrogallol solution and 0.5 to 0.1ml of enzyme extract in a test tube. Adjusted the spectrophotometer to read '0' at 400nm. Added 0.5ml of 1% H_2O_2 in the test cuvette. Recorded the change in the absorbance every 30 seconds up to 3 minutes.

Calculations

Change in absorbance / min	=	X
Weight of the plant material taken	=	250mg
Volume of the extract taken for the assay	=	0.1ml
Change in absorbance	=	X
Change in absorbance for 1.5ml extract	=	(X/0.1) × 1.50 – Y
(i.e) Peroxidase activity in 250mg plant tissue	=	Y
Peroxidase activity / gram of plant tissue	=	Y × (1000/250) Units.

APPENDIX XI
ESTIMATION OF SUPEROXIDE DISMUTASE ACTIVITY
(Misra and Fridovich, 1972)

Principle

Superoxide dismutase uses the photochemical reduction of riboflavin as oxygen generating systems and catalyses the inhibition of Nitro Blue Tetrozolium (NBT) reduction, the extent of which can de assayed spectrophotometrically.

Reagents

1. 50mM potassium phosphate buffer, (pH 7.8)
2. 45µM Methionine
3. 5.3µM Riboflavin
4. 84µM Nitro blue tetrozolium (NBT)
5. 20mM potassium cyanide

Procedure

The incubation medium contained a final volume of 3ml, 50mM potassium phosphate buffer (pH 7.8), 45μM Methionine, 5.3μM Riboflavin, 84μM Nitro blue tetrozolium (NBT), and 20mM potassium cyanide. The tubes were placed in aluminum Foil – lined box maintained at 25°C and equipped with 15W fluorescent lamps. Reduced NBT was measured spectrophtometrically at 600nm after exposure to light for 10 minutes. The maximum reduction was evaluated in the absence of the amount of enzyme giving 50% inhibition of the reduction of NBT.

APPENDIX XII
ASSAY OF GLUTATHIONE – S- TRANSFERASE
(Habig *et al.*, 1974)

The enzyme was assayed by the conjugate GSH and CDNB, the extent of conjugation causing a proportionate change in the absorbance at 340nm.

Reagents

1. 1mM - Chloro 2,4-dinitrobenzene (CDNB) in ethanol
2. 1mM - Glutathione
3. 0.1M Phosphate buffer

Procedure

The assay was done at 5°C under condition giving activities linear with respects to incubation time and protein concentration for at least 3 minutes. The enzyme activity was determined by monitoring the change in absorbance at 340nm in a spectrophotometer. 0.1ml of both substrates GSH and CDNB was taken in 0.1M phosphate buffer (pH 6.5) at room temperature to make a volume of 2.9ml. The reaction was started by the addition of 0.1ml of sample to this mixture; the readings were recorded against distilled water blank for a minimum of three minutes. The complete assay mixture without the sample served as the control to monitor non-specific binding of the substrate. It was taken to ensure that final concentration of ethanol in the mixture was always less than 4%.

Calculation

GST activity was calculated using the extinction coefficient of the product formed and the values have been expressed as nmoles and CDNB conjugated/minutes/mg of protein.

APPENDIX XIII
ASSAY OF GLUTATHIONE PEROXIDASE
(Rotruck *et al.*, 1973)

A known amount of enzyme preparation was used to react with H_2O_2 in the presence of GSH for a specified time period. Then the remaining GSH was mesured by the method of Ellman.

$$2\ GSH + H_2O_2 \xrightarrow{\text{Se- GPX}} GSSH + 2\ H_2O_2$$

Reagents

1. 0.4M tris buffer pH 7.0
2. 10mM sodium azide
3. 10% TCA
4. 0.4mM EDTA

5. 2mM Glutahione

Procedure

To 2ml of tris buffer, 0.2ml of EDTA, 01ml of sodium azide and 0.5ml of plant extract were added. 0.2ml of glutathione followed by 0.1ml of hydrogen peroxide were added to the mixture, mixed well and incubated at 37°C for 10 minutes along with a tube containing all the reagents except sample. After 10 minutes the reaction was arrested by the addtion of 0.5ml of 10% TCA , centrifuged and the supernatant was assayed for glutathine by the method of Ellman. The activities are expressed as µg of GSH consumed/ minute/ mg of protein.

APPENDIX XIV
ESTIMATION OF ASCORBIC ACID
(Roe and Kuether, 1953)

Principle

Ascorbate is converted to dehydroascorbate by treatment with activated charcoal and bromine. Dehydroascorbic acid then reacts with 2, 4- dinitrophenyl hydrazine to form osazones, which dissolves in sulpuric acid to give an orange coloured solution whose absorbance can be measured spectrophtometrically at 540nm.

Reagents

1. 1% TCA
2. 9 N H_2SO_4
3. **2% 2, 4-dinitrophenyl hydrazine**: dissolved 2g of DNPH in 100ml of 9N H_2SO_4
4. 10% thiourea
5. 80% sulpuric acid
6. **Stock standard solution**: Dissolved 100mg of ascorbic acid in 100ml 4%TCA
7. **Working standard**: Diluted 10ml of the stock solution to 100ml with 4%TCA

Procedure

About 1g of the sample was homogenized in 4% TCA up to 10ml. Centrifuged at 2000rpm for 10 minutes. To the supernatant obtained, a pinch of activated charcoal was added, shaken well and kept for 10 minutes. Centrifuged once again and removed the charcoal residue. The volume of the clear supernatants was noted. 0.5 and 1.0 ml aliquots of this supernatant were taken for the assay. The assay volumes were made up 2.0ml with 4%TCA. 0.2 to 1.0ml of the working standard solution containing 20-100 µg of ascorbate respectively were pipetted out into clean dry test tube, the volume of which were also made up to 2.0ml with 4%TCA. Added 0.5ml of DNPH reagent to all the test tubes, followed by 2 drops of 10% thiourea solution. Incubated at 37°C for 3 hours. The osazones formed were dissolved in 2.5ml of 85% sulpuric acid, in cold, drop by drop, with no appreciable rise in temperature. To the blank alone, DNPH reagent and thiourea were added after the addition of H_2SO_4. The tubes were incubated for 30 minutes at room temperature, and the absorbance was read spectrophtometrically at 540nm. Calculated the content of ascorbic acid in the sample using the standard graph.

APPENDIX XV
ESTIMATION OF TOCOPHEROL
(Rosenberg, 1992)

Principle

Tocopherol can be estimated using Emmerie – Engel reaction which based on the reduction of ferric to ferrous ions by tocopherols, which then forms a red colour with 2, 2'-dipyridyl. Tocopherol and carotenes are first

extracted with xylene and the extraction read at 460nm to measure carotenes. A correlation is made for these after adding ferric chloride and reading at 520nm.

Reagents

1. Absolute alcohol
2. Xylene
3. 2, 2'- dipyridyl
4. **Standard solution**: Dissolved 10mg/ 10ml of œ-tocopherol in absolute alcohol 91mg of œ- tocopherol is equivalent to 100mg of tocopherol acetate.

Extraction

The sample was homogenized with water in a blender. Weighed accurately 2.5g of the homogenized sample into a conical flask. Added 50ml of 0.1N H_2SO_4 slowly without shaking. Stoppered and allowed to stand overnight. The next day contents of the flask were shaken vigorously and filtered through whatmann No.1 filter paper, discarding the initial 10-15ml of filtrate. Aliquots of the filtrate were used for the estimation.

Procedure

Into 3 stoppered centrifuge tubes (test, standard and blank), pipetted out 1.5ml of extract, 1.5ml of standard, 1.5ml of water respectively. To the test and blank added 1.5ml of ethanol and to the standard, added 1.5ml of water. Added 1.5ml xylene to all the test tubes, stoppered, mixed well and centrifuged. Transferred 1.0ml of xylene layer into another stoppered tube, taking care not to include any other ethanol or protein. Added 1.0ml of 2, 2'- dipyridyl reagent to each tube, stoppered and mixed. Pipetted out 1.5ml of the mixture into colorimeter cuvettes and read the extinction of the test and standard against the blank at 460nm. Then in turn beginning with the blank, added 0.33ml of ferric chloride solution. The amount of vitamin E can be calculated using the formula,

$$\text{Amount of tocopherols in } \mu g = \frac{\text{Reading at 520nm} - \text{Reading at 460nm}}{\text{Reading of standard at 520nm} \times 0.29 \times 15}$$

APPENDIX XVI
ESTIMATION OF REDUCED GLUTATHIONE
(Moron *et al.*, 1979)

Principle

Reduced glutathione (GSH) is measured by its reaction with DTNB (5, 5-dithio- 2 -nitrobenzoic acid) (Ellman's reaction) to give a compound that absorbs at 412nm.

Reagents

1. DTNB
2. 5%TCA
3. 0.2M Sodium phosphate buffer

Procedure

1g of the sample was homogenized in 5%TCA to give a 20% homogenate. The precipitated protein was centrifuged at 1000rpm for 10 minutes. The homogenate was cooled on ice and 0.1ml of supernatant was taken for the estimation. The volume of the aliquot was made up to 1.0ml with 0.2M sodium phosphate buffer (pH 8.0), 2ml of freshly prepared DTNB solution (0.6mM) in 0.2M phosphate buffer (pH 8.0), was added to the tubes and

intensity of the yellow colour formed was read at 412nm in a spectrophotometer after 10 minutes. A standard curve of GSH was prepared using concentration ranging from 2 to 10 nmoles of GSH in 5%TCA.

APPENDIX XVII
ANALYSIS OF AMYLASE ACTIVITY
(Peter, 1955)

Principle

The reducing sugar produced by the action of α and β amylase react with dinitrosalicylic acid and reduced it to a brown color product, nitro amino salicylic acid.

Reagents

1. **Sodium acetate buffer:** 0. 1 M, pH 4.7
2. **1% starch solution:** A fresh solution of starch was prepared by dissolving 1 g of starch in 100ml sodium acetate buffer (slightly warm if necessary).
3. **Dinitrosalicylic acid reagent (DNS reagent):** By continuous stirring, 1g Dinitrosalicylic acid, 200mg crystalline phenol and 50 mg sodium sulphate in 100ml 1%NaOH were dissolved and stored at 4°C.
4. 40% Rochelle salt solution (Potassium sodium tartarate)
5. **Maltose solution:** A quantity of 50mg of maltose was dissolved in 50ml of distilled water in a standard flask and stored it in a refrigerator.

Extraction of α Amylase

A quantity of 1g of sample material was extracted in 5-10 volumes of ice-cold 10mM calcium chloride solution overnight at 4°C or for 3hrs at room temperature. The extract was centrifuged at 54,000g at 4°C for 20mins.the supernatant was used as enzyme source.

Procedure

About 4ml of starch solution and 1ml of properly diluted enzyme were pipetted out in a test tube. It was incubated at 27°C For 15mins and then the reaction was stopped by the addition of 2ml of Dinitrosalicylic acid reagent. The solution was heated in a boiling water bath for 5mins. About 1ml of Potassium sodium tartarate solution was added while the test tubes were warm. Then it was cooled in a running tap water. The volume was made up to 10ml with distilled water. The absorbance was read at 560nm and the reaction was terminated at zero time in the control tube. A standard graph was prepared with 0-100mg of maltose.

Calculation

The activity of α - Amylase was calculated as μg maltose produced g^{-1}.

APPENDIX XVIII
ANALYSIS OF CELLULASE ACTIVITY
(Sumner.1955)

Principle

Cellulase acts on cellulose and breaks it down into simpler sugars. The activity of enzyme cellulase was estimated by calculating the amount of glucose released as a result of cellulose action.

Reagents

1. **Copper reagent A**: 25g of sodium carbonate, 25g of Rochelle salt, 20g of sodium bicarbonate and 200g of sodium sulphate was added to 800ml of distilled water and diluted to 1litre.

2. **Copper reagent B**: 15% $CuSO_4.7H_2O$ containing 1 or 2 drops of concentrated sulphuric acid was added to 100ml of distilled water.
3. **Arsenomolybdate color reagent**: 25g of ammonium molybdate were dissolved in 450ml of distilled water to which 21ml of concentrated sulphuric acid was added and mixed. 3g of sodium arsenate was dissolved in 25ml of water and placed in incubator at 37°C for 24-48 hrs.
4. **Standard glucose solution**: 100mg of glucose was dissolved in 100ml of distilled water.
5. Toluene
6. Phosphate buffer pH 5.8
7. 1% Carboxy methyl cellulose

Extraction

To 1g of soil sample, 1ml of phosphate buffer, 2drops of toluene, 1ml of Carboxy methylcellulose and 1ml of distilled water were added in Erlenmeyer flask. The flask was stoppered and incubated at 30°C for 24hrs, the supernatant was used for estimation of reducing sugars.

Procedure

To 1ml of supernatant, added 1ml of copper reagent-A and copper reagent-B mixture (25:1). The tubes were kept in boiling water bath for 20mins. After cooling under the tap water, 1ml of Arsenomolybdate reagent was added. The mixture was diluted to 25ml with distilled water. After 15 mins the absorbance was measured colorimatrically at 500nm. Standards and blank were treated similarly.

APPENDIX XIX
ANALYSIS OF DEHYDROGENASE ACTIVITY
(Chendrayan *et al.*, 1980)

Reagents

1. **3% 2, 3, 5- Tri phenyl tetrazolium chloride**: 3g of 2, 3, 5- Tri phenyl tetrazolium chloride dissolved in 100ml of distilled water.
2. **1%glucose**: 1g of glucose dissolved in 100ml of distilled water.
3. Methanol.

Procedure

To 5g of soil sample, 1ml of 3% 2, 3, 5- Tri phenyl tetrazolium chloride, 1ml of 1% glucose and 2.5ml of distilled water were added in a boiling tube. The tubes were incubated for 24hrs at room temperature. Then added 10ml of methanol to that and allowed incubation for another 5hrs. Then the contents were filtered using whatmann No1 filter paper. The filterate was red in color. The absorbance was read at 485nm. Take distilled water as a blank.

Calculation

Concentration of dehydrogenase (X) was obtained from standard graph

Dehydrog enase activity of the sample (μg of TPF/g) = X/ W_1

Where as, W_1= sample weight

APPENDIX XX
ANALYSIS OF PHOSPHATASE ACTIVITY
(Tabatabai and Bremner, 1969)

Reagents

1. **0.5M sodium hydroxide**: Dissolved 10g of sodium hydroxide in 500ml of distilled water.
2. **10mM Para Nitro Phenyl Phosphate (PNPP):** Dissolved 0.371g of sodium hydroxide in 100ml of distilled water.

3.**0.5M Calcium chloride**: Dissolved 13.874g of sodium hydroxide in 250ml of distilled water.

Procedure

To 5g of soil sample, 0.25ml of toluene, 1ml of 10mM PNPP and 10ml of distilled water were added in a boiling tube. The tubes were incubated for 1hr at room temperature. Then added 5ml of 0.5M Calcium chloride and 20ml of 0.5M sodium hydroxide. Then the contents were filtered through whatmann No1 filter paper. The filterate was make upto 50ml with water. The absorbance was read at 420nm. Take distilled water as a blank.

Calculation

Concentration of Phosthatase (X) was obtained from standard graph

Phosphatase activity of the sample (μg of PNPP/g) = X/ W_1

Where as, W_1= sample weight

APPENDIX XXI
ANALYSIS OF UREASE ACTIVITY
(Sumner, 1955)

Principle

Urease catalyses the hydrolysis of urea to CO_2 and NH_3. This is then treated with Nessler's reagent and the yellow colour developed was read at 495 nm in a spectrometer.

Reagents

1. 3% urea solution

2. 10% TCA

3. **Nessler's reagent**: 10g of mercuric iodide and 7g of potassium iodide were dissolved in 50ml of water (solution I). 20g of NaOH was dissolved in 50ml of water (solution II). Solution I and II were mixed together and filtered.

Procedure

About 0.5ml of buffer, 0.1ml substrate and 0.1ml enzyme were incubated at 37°C for 15 minutes. The reaction was stopped by the addition of 0.2ml of 10% TCA. Along with theses, controls were also setup to which the enzyme was added at the end of the incubation period. It was allowed to stand for 30 minute and centrifuged. About 0.5ml of supernatant from each tubes was transferred to other tubes. Then 1ml of Nessler's reagent was added and the volume made up to 10ml with distilled water. The colour developed was read at 495nm after 5 minutes.

Calculation

The activity of urease was calculated as nanomoles of ammonia liberated per minute per mg of protein.

APPENDIX XXII
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

$$\text{Nitrogen Content (1\%)} = \frac{0.0028 \text{ X Titre value X } 100}{10} \text{ X } \frac{100}{0.5}$$

APPENDIX XXIII
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 XXIV
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 XXV
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 XXVI
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.