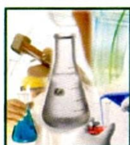
Appendices



APPENDICES

APPENDIX 1

DERMINATION OF pH

(ISI Bulletin, 1982)

Principle

The basic principle of electrometric pH measurement is dermination of the activity of the hydrogen ions by using a water analyzer.

Reagents

Buffer pH : 7 and 9.2

Procedure

1. 5 grams of dry sample was taken in a 100 ml beaker and 50 ml of distilled water was added. The contents were mixed well using a glass rod.
2. After 30 minutes, the pH was measured by using water analyzer 371 (Systronic). The instrument was calibrated by preparing buffer solutions of 7 and 9.2.
3. The sample pH was noted from the pH meter.

APPENDIX 2

DERMINATION OF ELECTRICAL CONDUCTIVITY

(ISI Bulletin, 1982)

Principle

The dermination of electrical conductivity (EC) is made with a conductivity cell by measuring the electrical resistance of 1:10 W/V sample: water suspension.

Reagents

1. Distilled or deionised water.
2. 0.01 M Potassium chloride reference solution: 0.746 g of potassium chloride (previously dried at 105°C for 2 hours) was dissolved in water and the volume was made upto one litre with distilled or deionised water.

Procedure

1. 5 grams of dry sample was taken in a 100 ml beaker and 50 ml of distilled water was added.
2. The content was mixed well using a glass rod.
3. After 30 minutes, the electrical conductivity was determined
4. The conductivity meter was calibrated using potassium chloride reference solution to obtain the cell constant.
5. The cell was rinsed thoroughly and the electrical conductivity of the 0.01 M potassium chloride was measured at the same temperature as the sample suspensions.
6. The conductivity cell with the sample suspension was rinsed.
7. The conductivity cell was refilled without disturbing the settled sample.
8. The value indicated on the conductivity meter was recorded.
9. The electrode was rinsed with deionised water between samples.
10. The electrical conductivity of the sample was expressed as (ds m^{-1})

APPENDIX 3

DERMINATION OF ORGANIC MATTER AND ORGANIC CARBON

(Modified Walkley-Black Procedure)

Principle

Organic matter is oxidized with a mixture of potassium dichromate and sulphuric acid. Unused potassium dichromate is back titrated with ferrous sulphate. The dilution heat of concentrated sulphuric acid with potassium dichromate is the sole source of heat. Because no external source of heat is applied, the method provides only an estimate of readily oxidizable organic carbon and is used as a measure of total organic carbon. Organic matter is estimated on the assumption that organic matter contains 58 % carbon (i.e Van Bemmelon factor). However, this percentage varies considerably from sample to sample. Because of the problems associated with organic matter determination, it is recommended that researchers determine and report the organic carbon content as a measure of the organic matter in a sample (Nelson and Sommers, 1982).

Reagents

1. Standard potassium dichromate solution (0.1667M=1 N): 49.04 g of potassium dichromate (dried at 105^0 C for 2 hours) was dissolved in distilled water and made up to one litre with distilled water.
2. Ferrous sulphate solution (0.5 N): 140 g of reagent grade ferrous sulphate was dissolved in about 800 ml water and 40 ml concentrated sulphuric acid cooled and diluted to 1 litre in a volumetric flask and mixed thoroughly. The solution was kept in a tightly stoppered bottle. Standardization was done daily by titrating against standard dichromate solution (this is the blank)
3. Ortho-phenenthroline ferrous sulphate (0.025 M) indicator solution, available under the trade name ferroin was used directly at this strength.
4. Sulphuric acid, concentrated, not less than 96 % (specific gravity 1.84)

Procedure

1. 5 g of sample was ground using a mortar and pestle (Iron and steel mortar should not be used).
2. About 0.5 g of air dried sample was taken into a 125 ml- Erlenmeyer flask.
3. Two blanks were included to standardize the ferrous sulphate solution.
4. To this 10 ml of potassium dichromate solution 20 ml of sulphuric acid was added and incubated for half an hour.
5. About 30 ml of distilled water and 3-4 drops of indicator were added.
6. The mixture was shaken thoroughly and titrated against the burette solution of ferrous sulphate till the end point became dull green to greenish blue in colour.
7. At this point, the titrant was added dropwise and the solution flashes quickly from greenish blue to reddish brown.
8. The titration completion was checked by adding a drop of dichromate solution.
9. The colour should change back to greenish blue (burette should have one drop accuracy).
10. If the end point was over run a small volume of dichromate solution (recorded the amount added) was run and the titration was completed. Titration with distilled water was carried out as blank.

11. If burette reading of ferrous sulphate was 0-4 ml, repeated with less sample, if it was 17 ml or higher, repeated with more sample.

Calculation

$$\text{Organic Carbon (g \%)} = M \times \frac{V_1 - V_2}{\text{Weight of the sample (g)}} \times 0.39,$$

where

M = molarity of the ferrous sulphate solution

V_1 =volume of the ferrous sulphate solution used against blank (ml).

V_2 = volume of the ferrous sulphate solution used against the sample (ml).

$0.39 = 3 \times 10^{-3} \times 100 \times 1.3$, where 3 is the equivalent weight of carbon and 1.3 is the factor explained below

There is incomplete oxidation of the organic matter in this procedure. The factor of 1.3 is based on the assumption that there was 77 % recovery.

Organic matter (g %) = organic carbon (g %) × 1.724

The Van Bemmelen factor of 1.724 is used because organic matter contains 58 % carbon.

APPENDIX 4

DERMINATION OF TOTAL NITROGEN

(Microkjeldahl method –Tandon, 1993).

Principle

The nitrogen content of the sample is converted into ammonium sulphate by boiling with concentrated sulphuric acid. It is subsequently decomposed by addition of excess of alkali and the liberated ammonia is absorbed into boric acid solution containing bromocresol and methyl red indicators by which the liberated ammonia forms a loose compound, ammonium borate with boric acid which is titrated against 0.1 N sulphuric acid.

Reagents

1. 10 % Sodium hydroxide

2. 2 % Boric acid solution

3. 0.1 N Standard sulphuric acid

4. Bromocresol green and methyl red indicators

Procedure

Digestion of the sample

This was carried out using a 7:3 mixture (713 ml: 286 ml) of sulphuric acid plus perchloric acid. One gram of the dry sample was placed in a 100 ml volumetric flask. To this, 10 ml of diacid mixture was added and the contents of the flask were mixed by swirling. The flask was then placed on the hot plate and at a higher temperature until the production of red nitrate fumes ceases. After digestion, the flask was cooled and the sample was diluted with double distilled water in a 100 ml standard flask. The solution was then filtered through Whatman No.1 filter paper. This digested solution was used for all the elemental analysis.

Distillation by Kjeldahl method

1. To the vacuum jacket of the Micro-kjeldahl distillation apptus, 10 ml of the digest was transferred and 10 ml of 10% sodium hydroxide was added.
2. In a 100 ml beaker, 2 ml of boric acid solution was taken containing bromocresol green and methyl red indicators, to which the condenser outlet of the flask was dipped.
3. After completion of distillation, the colour of the solution changed into green. Then this solution was titrated against 0.1 N standard sulphuric acid, the appearance of pink colour was the end point.

Calculation

weight of the substrate used in the extraction = 1 g

volume of digestion =100 ml

aliquot taken =10 ml

0.1N sulphuric acid is equivalent to = 0.0014 g Nitrogen

$$\text{Total N (g \%)} = \frac{\text{Titre value} \times 0.0014 \times 100 \times 100}{1 \times 10}$$

$$= \text{Titre value} \times 1.4$$

APPENDIX 5

DERMINATION OF TOTAL PHOSPHORUS

(Tandon, 1993)

Principle

Ammonium molybdate reacts under acid conditions to form a heteropolyacid molybdophosphoric acid. In the presence of vanadium, yellow vanadomolybdophosphoric acid is formed. The intensity of the yellow colour is proportional to the phosphate concentration. Concentration ranges for different wavelengths are

Phosphorus range (mg/litre)	Wave length (nm)
1.0-5.0	400
2.0-10	420
4.0-18	470

Reagents

1. Solution A: 25 g of ammonium molybdate was dissolved in 300 ml warm distilled water and cooled.
2. Solution B: 1.25 g of ammonium metavanadate was dissolved in 300 ml boiled distilled water and cooled. 250 ml concentrated nitric acid was added. The solution B was cooled and mixed with solution A and made upto one litre.
3. Standard phosphorus solution: 0.2195 g of dried potassium dihydrogen orthophosphate was dissolved in distilled water; acidified with 25 ml of 7 N sulphuric acid and made upto one litre to get 50 mg/litre phosphate solution.

Procedure

1. 10 ml of acid digests of samples were placed in a 50 ml volumetric flask, 10 ml of the vanadate-molybdate reagent was added and diluted to 50 ml and mixed well.
2. The phosphorus concentration was read after 10 minutes using a spectrophotometer at 420 nm.
3. 0, 1, 2, 3, 4 and 5 ml of the 100 mg/litre phosphorus solution were taken in a 50 ml volumetric flask and the colour developed in an identical manner.

4. The spectrophotometer was calibrated with known phosphorus concentration and the concentration of phosphorus in the sample was calculated.

Calculation

$$P\ \mu g/g = \frac{R \times 50}{10} \times \frac{100}{\text{Sample wt. (g)}}$$

R = reading of spectrophotometer phosphorus mg/l.

10 = volume of acid digest used for colour development.

50 = volume made up for colour development.

100 = volume made up after acid digestion.

g = sample wt. (g) for acid digestion.

APPENDIX 6

DERMINATION OF TOTAL POTASSIUM, SODIUM AND CALCIUM

Flame Photometric Method

(Tandon, 1993)

Principle

The sample in solution is introduced in the form of a fine continuous spray into a non luminous gas flame. After evaporation of the water, the salt molecules are dissociated by the heat to an atomic vapour. A small percentage of atoms are transformed to an excited state by the absorption on discrete packets of energy that displace orbital electrons to higher energy levels. The atoms immediately return to the ground state and release the absorbed packets of energy in the form of light. The emitted light is of wavelength specific to each element and can be determined under carefully controlled conditions.

Reagents

1. Standard stock solution of potassium

To prepare a standard stock solution of potassium in a 1000 ml standard flask, 1.91 g of potassium chloride was dissolved in distilled water and made upto one litre. This solution contains 1000 ppm of potassium. From this stock 10, 20, 30, 40, and 50 ppm of potassium solutions were prepared by appropriate dilutions.

2. Standard stock solution of sodium

To prepare a standard stock solution of sodium, in a 100 ml standard flask, 2.54 g of sodium chloride was dissolved in distilled water and made up to one litre. This solution contains 1000 ppm of sodium. From this stock 5, 10, 15 and 20 ppm of sodium solutions were prepared by appropriate dilutions.

3. Standard stock solution of calcium

To prepare a standard solution of calcium 2.25 g of calcium carbonate was dissolved in 5 ml of deionized water in a 1000 ml standard flask. Approximately 10 ml of hydrochloric acid was added dropwise to dissolve completely the calcium carbonate, then diluted to 1 litre with deionized water. This solution contains 1000 ppm of calcium.

Procedure

1. As per the operation manual the filter for specific elements on Flame Photometer was set. Air pressure was kept at 15 1bs.
2. Standard solutions of the highest value in the standard series were fed and adjusted the flame photometer to read full value of emission in the scale (100 reading). The zero reading of the meter was adjusted by feeding distilled water.
3. Now different standard solutions were fed one by one and recorded the emission value of each. A standard curve was plotted between concentration and readings of the standard solutions.
4. 10 ml aliquot was pipetted out into a 100 ml volumetric flask and diluted to the mark with distilled water.
5. The diluted aliquot was fed to the flame photometer and recorded the readings. With the help of standard curve the concentration of the aliquot was noted.

Calculations

volume of extract = 100 ml

aliquot taken = 10 ml

$\frac{100}{1}$ = dilution factor

$$\text{K, Na and Ca (g \%)} = \frac{a}{10^6} \times \frac{100}{10} \times \frac{100}{1} \times 100$$

APPENDIX 7

DERMINATION OF AVAILABLE NITROGEN

(Subbiah and Asija, 1956)

More than 90 % of nitrogen exists as complex organic compounds which the plants cannot use directly. It becomes available to plants after its mineralization. The easily mineralizable nitrogen is estimated using alkaline potassium permanganate, which oxidizes and hydrolyses the organic matter present in the sample. The liberated ammonia is condensed and absorbed in boric acid, which is titrated against standard acid.

Reagents

1. 0.32 % potassium permanganate: 3.2 g of potassium permanganate was dissolved in distilled water and made upto one litre with distilled water.
2. 2.5 % Sodium hydroxide: 25 g of sodium hydroxide was dissolved in distilled water and made upto one litre with distilled water.
3. 2 % Boric acid: 20 g of boric acid powder was dissolved in warm distilled water and made upto one litre with distilled water.
4. Mixed indicator: 70 mg of methyl red and 100 mg of bromocresol green were dissolved in100 ml of ethyl alcohol. 10ml of this mixed indicator was added to each litre of 2 % boric acid solution. The pH was adjusted to 4.5 using dilute hydrochloric acid or dilute sodium hydroxide.
5. 0.01 N Sulphuric acid: 0.1 N sulphuric acid was prepared by adding 2.8 ml of concentrated sulphuric acid to about 990 ml of distilled water. It was standardized against 0.1 N standard sodium hydroxide solution.

Procedure

1. 10 g of sample was weighed into a 250 ml Kjeldhal tube. To this about 50 ml of 0.32 % potassium permanganate solution and 50 ml of 2.5% sodium hydroxide solution was added and distilled immediately.

2. The distillate was collected in 10 ml of 2 % boric acid solution and titrated against 0.01 N sulphuric acid solution until pink colour started appearing.
3. A blank without sample was done for each set of samples.

Calculation

$$\text{Available nitrogen in the sample (mg/kg)} = \frac{(S-B) \times N \times 14}{\text{Sample weight (g)}} \times 1000$$

Where

S= volume of acid used against sample.

B= volume of acid used against blank.

N= normality of acid.

APPENDIX 8

DERMINATION OF AVAILABLE PHOSPHORUS

(Olsen *et al.,* 1954)

Principle

Phosphate in the sample is extracted using sodium bicarbonate solution of pH 8.5 in an acidic ammonium fluoride solution. After the extraction, the phosphate is determined colourimetrically with ammonium molybdate as the colouring reagent. This method is used for alkaline, calcareous or neutral samples.

Reagents

1. Sodium hydroxide (1 M): 4 g sodium hydroxide was dissolved in 100 ml of distilled water.
2. Sodium bicarbonate (0.5 M): 42 g sodium bicarbonate was dissolved in one litre water and the pH was adjusted to 8.5 with 1 M sodium hydroxide (the pH was checked every day).
3. Sulphuric acid (4 M): 56 ml concentrated sulphuric acid was added to 150 ml water and allowed to cooled and made upto 250 ml with water.
4. Ammonium molybdate (4 %): 4 g Ammonium molybdate was dissolved in 100 ml water and stored in a polythene bottle in dark.

5. Potassium antimony tartrate (0.275 %): 275 mg potassium antimony tartrate was dissolved in 100 ml water.
6. Ascorbic acid (1.75 %): 1.75 g ascorbic acid was dissolved in 100 ml water. (Prepared fresh daily).
7. Mixed reagent: the following reagents were added to a 500 ml bottle; 50 ml 4 M sulphuric acid, 15 ml 4 % ammonium molybdate, 30 ml 1.75 % ascorbic acid, 5 ml 0.275 % potassium antimony tartrate and 200 ml water. These solutions were mixed well after each addition and prepared freshly.
8. Standard series of phosphorus: 4.3943 g potassium dihydrogen orthophosphate (dried at 105^0C) was weighed and transferred into a 250 ml beaker; 200 ml water was added and made to dissolve. The volume was made upto one litre to get 1000 ppm standard phosphorus solution.
9. 10 ml of the 1000 ppm stock solution was pippeted out into a 100 ml volumetric flask. This made a 100 ppm phosphorus solution.
10. 10 ml of this 100 ppm phosphorus solution was pipette out into a 250 ml volumetric flask. This made a 4 ppm Phosphorus solution.
11. 0, 5, 10, 15, 20, and 25 ml of 4 ppm phosphorus solution was taken into a 50 ml volumetric flask and each flask content was upto volume with extracting solution to get a series of standards containing 0.0, 0.4, 0.8, 1.2, 1.6 and 2.0 ppm phosphorus.

Procedure

1. 5 g of sample was weighed and transferred into a 250 ml shaking bottle.
2. To this100 ml of sodium bicarbonate extractant was added and shaken for 30 minutes. It was filtered through Whatman no: 42 filter paper.
3. 5 ml of the standard series, sample or blank was taken in a test tube and 5 ml of mixed reagent was added.
4. The above tubes were shaken and allowed to stand for one hour for the blue colour development. The concentration of the solution was measured at 882 nm or 720 nm, using a spectrophotometer.

5. Initially, the spectrophotometer was standardized with a series of known concentrations and after that the phosphorus concentration in the sample was determined.

Calculation

$$\text{Available P (mg/kg)} = \frac{(S-B) \times D \times [100 + \{W - \frac{W}{(mcf)}\}]}{W\ (g)} \times mcf$$

S = phosphorus concentration in sample (mg/l) read by spectrophotometer.

B = phosphorus concentration in blank (mg/l) read by spectrophotometer.

D = dilution factor (standard 1 for undiluted samples).

W = weight of the sample.

mcf = moisture correction factor.

100 = volume of extractant.

APPENDIX 9

DERMINATION OF AVAILABLE POTASSIUM

(Hanway and Heidel, 1952).

Potassium in sample exists as water soluble, exchangeable and fixed. The first two forms constitute only a small part (not more than 1%) and are considered to be easily available to plants. These forms are determined by ammonium acetate method.

Apparatus

Flame photometer, Shaker, pH meter.

Reagents

1. Ammonium acetate (1 N): 77.08 g of ammonium acetate was dissolved in 500 ml of distilled water and made upto to one litre with distilled water. The pH was adjusted to 7.0 with glacial acetic acid or ammonia solution.
2. Standard potassium solutions: 1.908 g of oven dried potassium chloride salt was dissolved in one litre distilled water to get 1000 ppm potassium solution. This solution was diluted to get solutions of 10, 25, 50, 75 and 100 ppm potassium in ammonium acetate working solution.

Procedure

1. 5 g of sample was taken in a 100 ml conical flask and 25 ml 1 N ammonium acetate solution was added and shaken for 5 minutes.
2. The solution was filtered through Whatman No:1 filter paper and the potassium concentration in the filtrate was measured in a Flame Photometer.

Calculation

$$\text{Available K (mg/kg)} = \frac{C \times 25}{\text{Sample weight (g)}} \times \text{mcf}$$

where,

C = concentration of potassium in filtrate

mcf =moisture correction factor.

25 = volume of ammonium acetate.

APPENDIX 10

DERMINATION OF SULPHUR

(Raghuramulu *et al.,* 1983)

Principle

Sulphur is precipitated with Barium chloride and is estimated gravimetrically.

Reagents

1. 0.1 % Methyl orange
2. 40 % Sodium hydroxide
3. 5 % Barium chloride

Procedure

1. 2 g of sample was weighed into a microkjeldahl flask and 5 ml of concentrated nitric acid was added. The flask was covered with a glass stopper.
2. The flask was then kept in a boiling water bath for 10 hours and then on a sand bath and digested on a low flame till a liquid digest results (additional nitric acid was added if necessary).
3. To this 2 ml of perchloric acid was added and the temperature was raised.
4. Addition of nitric acid and perchloric acid was repeated till the digest turned into a clear solution.

5. The digestion was continued for further 10-16 hours.
6. By this time all the nitric acid had escaped and the remaining clear solution was transferred into a 500 ml beaker by repeated washings with distilled water and made the volume upto 200 ml.
7. One or two drops of methyl orange indicator were added and the solution was neutralized with the addition of 40 % sodium hydroxide.
8. The solution was again acidified with hydrochloric acid and boiled for 5 minutes.
9. About 10 ml of 5 % barium chloride was added and the solution was boiled for five minutes.
10. The solution was kept overnight and then filtered through Whatman No.40-44 filter paper and washed with distilled water till free from barium chloride.
11. The precipitate alongwith the filter paper was kept in an oven for drying. The filter paper was then taken in a weighed crucible and ignited, taking care to avoid spurting and finally ashed at 600^0 C for 3-4 hours and weighed.

Calculation

$$\text{Sulphur (g \%)} = \text{Wt of the ash} \times 0.1374 \; \frac{100}{\text{Wt of the sample}}$$

APPENDIX 11

DERMINATION OF TRACE ELEMENTS
(Copper, Iron, Magnesium, Molybdenum, Zinc, Boron)

Atomic Absorption Spectrophotometric method (Krishna and Ranjan,1991)

Principle

The technique involves dermination of concentration of a substance by the measurement of absorption of the characteristic radiation by the atomic vapour of an element. When radiation characteristic of a particular element passes through an atomic vapour of the same element, absorption of radiation occurs in proportion to the concentration of atoms in the light path. The source of characteristic radiation is a hollow cathode lamp being made of the element desired to be estimated.

Reagents

1. Triple acid mixture- concentrated nitric acid - perchloric acid-concentrated sulphuric acid (3:2:1)

Procedure

1. 5 g of the dry sample was weighed and 25 ml of 3:2:1 triple acid mixture was added and left aside for 3-4 hours in a fume cupboard.
2. It was then heated for 30 minutes until the initial vigorous reaction was subsided.
3. It was heated more strongly for 4 hours until the nitrous fumes were removed and white fumes of perchloric acid were evolved.
4. The contents were allowed to cool and transferred with 3-4 washings of deionised water to a 50 ml volumetric flask and made upto the mark with water. Aliquots of the sample were taken for the estimation of heavy metals in an Atomic Absorption Spectrophotometer.

APPENDIX 12

DERMINATION OF CELLULASE ACTIVITY

(Schinner, 1990; Denision and Koehn, 1977)

Principle

The production of reducing sugar by cellulase activity is measured by dinitrosalicylic acid reagent at 540 nm.

Reagents

1. Sodim citrate buffer 0.1M (pH 5.0)
2. Carboxymethyl cellulose (CMC) reagent 0.7 %: 0.7 g carboxmethyl cellulose was dissolved in 100 ml sodium citrate buffer 0.1M (pH 5.0)
3. Dinitrosalicylic acid (DNS) reagent: 1 g DNS, 200 mg crystalline phenol and 50 mg sodium sulphate were dissolved in 100 ml 1 % sodium hydroxide and stored at 4^0C.
4. 40 % Rochelle salt solution (Potassium sodium tartarate)
5. Acetate buffer pH 5.5

Sample preparation

1 g of sample was incubated with 10 ml of 2 M acetate buffer (pH 5.5) containing the substrate, carboxymethyl cellulose sodium salt (0.7 % w/v) for 24 hours at 50°C (Schinner, 1990). The reducing sugar released in the filtrate was analyzed by using DNSA reagent according to the procedure given by Denision and Koehn, 1977.

Procedure

1. 0.5 ml of the enzyme - substrate mixture was added to 0.5 ml DNS reagent.
2. The mixture was heated in a boiling water bath for 5 minutes. While the tubes were warm, 1.0 ml potassium sodium tartarate solution was added.
3. The tubes were cooled to room temperature. To this water was added to make the volume 5 ml. The absorbance was measured at 540 nm.
4. A standard graph was prepared by taking glucose in the concentration range 50 μg to 1000 μg/ml.

Calculation

The enzyme activity was expressed as the μg of reducing sugar released g^{-1}dry weight 24 h^{-1}.

APPENDIX 13

DERMINATION OF β-GLUCOSIDASE ACTIVITY

(Eivazi and Tabatabai, 1988)

Principle

Activity of β-glucosidase was determined by incubation of the sample with p-nitrophenyl glucoside for 1 hour at 37° C. The released p- nitrophenol (PNP) was read at 405 nm spectrophotometrically by using calcium chloride and tris buffer.

Reagents

1. Modified Universal Buffer (MUB) stock solution: 12.1 g of tris hydroxymethyl aminomethane (THAM), 11.6 g of maleic acid, 14.0 g of citric acid, 6.3 g of boric acid (H_2BO_3), and 0.488 ml of 0.1 N sodium hydroxide were dissolved and made the solution to 100ml with water. It was stored in refrigerator

2. Modified Universal Buffer (MUB) pH 6: 200 ml of MUB stock solution in a 500ml was titrated with 0.1 M hydrochloric acid to get pH 6 and the volume was adjusted to 1litre with water.
3. 0.5 M p- nitrophenyl-β-D-glucopyranoside substrate solution
4. 0.5 M calcium chloride
5. P-nitrophenyl phosphate solution 0.05M: 0.84 g of sodium p-nitrophenylphosphate tetrahydrate was dissolved in 40ml of MUB pH 6 and diluted to 50 ml with MUB of the same pH. The solution was stored in a refrigerator.
6. Tris buffer (pH 10)

Procedure

1. 1 g of the air dried sample was placed in a plastic tube and treated with 4 ml of Modified Universal Buffer (MUB, pH 6) and added 1 ml of 0.5 M p-nitrophenyl-β-D-glucopyranoside solution.
2. The solution was mixed thoroughly and allowed to incubate in the dark for 1 hour at 37°C.
3. After incubation, the solution was mixed and filtered through a 0.45 μm syringe tip filter.
4. To the filtrate 1 ml 0.5 M calcium chloride and 4 ml of tris buffer (pH 10) was added.
5. Controls were prepared by adding the substrate after the reaction was stopped.
6. Solutions of 0-5 μg p-nitrophenol (15 mM p-nitrophenol in MUB, pH 6) were made to develop a standard curve.
7. The yellow colour was developed by adding 1 ml of 0.5 M calcium chloride and 4 ml of tris buffer (pH 10) and measured spectrophotometrically at 405 nm.
8. The standard solutions were measured by absorption on a spectrophotometer at a wavelength of 405 nm and a standard curve was developed by plotting absorption vs concentration.

Calculation

The enzyme activity was expressed as the μg of paranitro phenol released g^{-1}dry weight h^{-1}

APPENDIX 14

DERMINATION OF PROTEASE

(Ladd and Butler, 1972; Mahadevan and Sridar, 1996).

The release of aromatic amino acids by protease activity can be measured by using Folin-phenol reagent at 700 nm.

Reagents

1. 2 % sodium caseinate
2. 5 % trichloroacetic acid
3. Folin-phenol reagent
4. 20 % sodium carbonate
5. 50mM Tris Buffer (pH 8.1)

Procedure

Sample preparation

1 g of the sample (1 g oven dried equvalent) was incubated with 5 ml of 50 mM tris Buffer (pH 8.1) and 5 ml of (2 %) sodium caseinate for 2 h at 50°C. The aromatic amino acids released were determined by the procedure explained by Mahadevan and Sridar 1996).

Procedure

1. 1 ml of the above enzyme - substrate mixture was taken in a centrifuge tube and 1 ml of 5 % trichloroacetic acid was added.
2. The mixture was allowed to stand for one hour and centrifuged at 2,000 g for 20 minutes. Pipetted out 1 ml of the supernatant into a test tube and added 1ml of Folin-phenol reagent and 2 ml of 20 % sodium carbonate.
3. Placed the tubes in boiling water bath for exactly 1minute.
4. Cooled under a running tap and increased the volume to 10 ml with distilled water.
5. The colour obtained was read at 650 nm.

6. The amount of amino acids released was calculated from a standard graph plotted from phenylalanine or tyrosine. Used reaction mixture with boiled enzyme and aliquots drawn at zero time were used as controls.

Calculation

The enzyme activity was expressed as µg of tyrosine released 2 hours $^{-1}$

APPENDIX 15

DERMINATION OF Urease

(Pancholy and Rice, 1973; Jackson, 1973)

The release of ammonia due to urease activity is measured colourimetrically at 410 nm by adding Nessler's reagent.

Reagents

1. Phosphate buffer (pH 7.6)
2. 10 % urea solution
3. 1 N potassium chloride solution
4. 10 % sodium potassium tartarate
5. Nessler's reagent
6. Standard ammonium sulphate solution concentrations (0- 100 µg /ml)

Procedure

1. In an Erlenmeyer flask, 10 g of sample was mixed with 1ml of toluene and allowed to stand for 15 minutes to permit complete penetration into the sample.
2. To the above 10 ml of phosphate buffer (pH 7.6) 10 ml of 10 % urea solution was added and incubated at 37° C for 24 hours.
3. After incubation, the contents of the flask were filtered through Whatman No: 42 filter paper.
4. The remaining sample in the flask was treated with 15 ml of 1N potassium chloride solution and shaken for five minutes and filtered.
5. The volume of the total filtrate was made upto 100 ml in a volumetric flask using distilled water. The amount of ammonia released can be estimated by the method described by Jackson, (1973) as explained below.

6. 2.5 ml of filtrate was transferred to a 50 ml volumetric flask to which 5 ml of 10 per cent sodium potassium tartarate solution and 1.5 ml of Nessler's reagent was added.
7. The volume was made upto 50 ml with distilled water and the yellow colour developed after 30 minutes was measured at 410 nm using a spectrophotometer against the blank.

Calculation

The results were expressed as µg of ammonia nitrogen liberated per gram sample (oven dry basis) per day with reference to a standard curve obtained by using graded concentrations (0- 100 µg /ml) of ammonium sulphate solution.

APPENDIX 16

DERMINATION OF DEHYDROGENASE

(Casida *et al.*, 1964)

Principle

The dermination of dehydrogenase activity involves colourimetric dermination of 2,3,5-triphenylformazan (TPF) produced by the reduction of 2,3,5-triphenyl tetrazolium chloride (TTC) by sample microorganisms. The colourless or pale coloured tetrazolium salt possesses the property of being easily transformed into intensely coloured, water insoluble, methanol soluble formazan by reduction. The intensity of the red colour was measured colourimetrically at 485 nm.

Reagents

1. Calcium carbonate ($CaCO_3$)
2. 3% 2, 3, 5-Triphenyl tetrazolium chloride (TTC): 3 g of TTC was dissolved in about 80 ml of water and the volume was made upto 100 ml with water.
3. Methanol

Procedure

1. 20 g of air dried sample was mixed with 0.2 g of calcium carbonate and 6 g of this mixture was placed in each of the three tubes.
2. To each tube, 1 ml of 3 % aqueous solution of TTC and 2.5 ml of distilled water were added.

3. This amount of liquid was sufficient such that a small amount of liquid appears at the surface of the sample after mixing.
4. The contents of each tube were mixed with a glass rod, closed and incubated at 37°C. After 24 hours, 10 ml of methanol was added and shaken for one minute.
5. The contents of the tubes were filtered through a glass funnel plugged with absorbent cotton into a 100 ml volumetric flask.
6. The tubes were washed with methanol and quantitatively and then added additional methanol (in 10 ml proportions) until the reddish colour has disappeared from the cotton plug.
7. The filtrate was diluted to 100 ml volume with methanol. The intensity of the reddish colour was measured by using a colorimeter at 485 nm. The absorbance was plotted against the standard values.

Calculation

The enzyme activity was expressed as μg of TPE released g^{-1}dry weight h^{-1}

Comment

The incubation should be anaerobic for 1-2 hours (or) for 24 hours (or) longer.

APPENDIX 17

DERMINATION OF PHOSPHATASES

(Tabatabai and Bremner, 1969)

Phosphatases are enzymes which catalyse the splitting of phosphoric acid from certain monophosphoric esters such as glycerophosphates and phenyl phosphate. Phosphatases are classified as acid and alkaline phosphatases because they show optimum activities in acid and alkaline ranges respectively.

Principle

The assay of phosphatases is based on the colourimetric estimation of the p-nitrophenol released by phosphatase activity when sample is incubated with buffer (pH 6.5 for acid phosphatase and pH 11 for alkaline phosphatase) sodium p-nitrophenyl phosphate solution and toluene. The phenols are yellow in colour in alkaline medium. Theinensity of the yellow colour was measured colourimetrically at 520 nm.

Apparatus required

1. Incubation flasks
2. Incubator
3. Colorimeter or spectrophotometer

Reagents

1. Toluene
2. Modified Universal Buffer (MUB) stock solution: 12.1 g of tris hydroxymethyl aminomethane (THAM), 11.6 g of maleic acid, 14.0 g of citric acid, 6.3 g of boric acid (H_2BO_3), and 0.488 ml of 0.1 N sodium hydroxide were dissolved and made to 100 ml with water. It was stored in a refrigerator
3. Modified Universal Buffer (MUB) pH 6.5 and 11: 200 ml of MUB stock solution was placed in a 500 ml beaker containing a magnetic stirrer. The solution was adjusted to pH 6.5 using 0.1 M hydrochloric acid, and the volume was made to 1 litre with water. Another 200 ml of the MUB stock solution was adjusted to pH 11 by using 0.1 M sodium hydroxide and the volume made to 1litre with water.
4. p-nitrophenyl phosphate solution 0.05 M: 0.840 g of sodium p-nitrophenylphosphate tetrahydrate was dissolved in about 40 ml of MUB pH 6.5 (for assay of acid phosphatase) or pH 11 (for assay of alkaline phosphatase), and the solution was diluted to 50 ml with MUB of the same pH. The solution was stored in a refrigerator.
5. Calcium chloride ($CaCl_2$) 0.5M: 73.5 g of $CaCl_2$ $2H_2O$ was dissolved in about 700 ml of water, and the volume was made to1litre with water.
6. Sodium hydroxide (Sodium hydroxide), 0.5 M: 20 g of sodium hydroxide was dissolved and made upto one litre with water. The solution was stored in a refrigerator.
7. Standard p-nitrophenol solution: 20 g of p-nitrophenol was dissolved in about 700 ml of water and made upto one litre with water. The solution was stored in a refrigerator.

Procedure

1. 1 g of the sample was placed in a 50 ml Erlenmeyer flask. To this 0.2 ml of toluene, 4 ml of MUB (pH 6.5 for assay of acid phosphatase or pH 11 for assay of alkaline phosphatase) and 1ml of p-nitro phenyl phosphate solution made in the same buffer were added and the flask was swirled for a few seconds to mix the contents.
2. The flask was stoppered and placed in an incubator at 37° C
3. After 1 hour 1 ml of 0.5 M calcium chloride and 4 ml of 0.5 M Sodium hydroxide, was added and the sample suspension was filtered through Whatmann No.2 filter paper.
4. The intensity of the yellow colour was measured in a colorimeter. The p-nitro phenol content of the filtrate was calculated from the standards containing 0, 10, 20, 30, 40 and 50μg of p-nitro phenol.

Calculation

The enzyme activity was expressed as μg of p- nitrophenol released g^{-1}dry weight h^{-1}

APPENDIX 18

DETERMINATION Of HUMIC ACID

(Valdrighi *et al.,* 1996)

The humic acid content of the sample can be determined by Alkali Fractionation Method

Reagents

1. 0.1 N potassium hydroxide
2. 6.0 N sulphuric acid

Procedure

1. The sample was digested in 0.1 N potassium hydroxide (1:10w/v) for 24 hours at room temperature.
2. The undigested bulk residue from the sample was then separated from the solute fraction by centrifugation at 5000 rpm for 30 minutes followed by vacuum filtration through a glass filter paper.

3. The filtered supernatant was acidified to pH 2 with 6.0 N sulphuric acid and kept in a cold room in the dark for 24 hours in order to obtain flocculation of humic acids.
4. After acidification, the humic precipitate (humate) was collected by centrifuging at 5000 rpm for 30 minutes and washed three times with distilled water to remove residual sulphuric acid.
5. The residue was freeze-dried and then ground with a mortar and a pestle into a brown powder.

APPENDIX 19

MICROBIAL ACTIVITY DETERMINATION THROUGH CARBONDIOXIDE EVOLUTION

(Anderson, 1982; Dubey and Maheswari, 2002)

Principle

Microorganisms present in the samples respire and evolve carbondioxide which can be measured and assessed as an index of microbial activity of sample.

Requirement

Sample	1 g
Conical flask (1 litre capacit)	2
Distilled water	100 ml
Sodium hydroxide solution	0.1 N
Hydrochloric acid solution	0.1 N
Saturated solution of barium chloride	
Phenophthalein dye	
Burette	

Procedure

1. 1 g of sample was weighed and transferred into a sterile flask (1 litre capacity).
2. It was mixed with distilled water to adjust sample moisture to its 33 % of water holding capacity.
3. 10 ml of freshly prepared 0.1 N sodium hydroxide solution was taken into two test tubes and the mouths of the test tubes were tied with a thread, and the tubes

were allowed to hang in the two flasks (the second devoid of sample acts as control) in such a way that the free end of the thread was out of the flask.

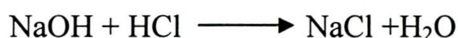
4. The mouth of the flasks were closed with rubber stopper and sealed with molten wax to make them air tight.
5. The contents of the flask were incubated at 30°C. The cardondioxide evolved as a result of microbial activity was absorbed by the 0.1 N sodium hydroxide present in the test tube and this was measured at specific intervals.

$$2NaOH + CO_2 \longrightarrow Na_2CO_3 + H_2O$$

6. At each interval the sodium hydroxide solution was transfered into a flask followed by addition of 5 ml of saturated barium chloride to get precipitated as barium carbonate.

$$Na_2CO_3 + BaCl_2 \longrightarrow BaCO_3 + NaCl$$

7. The residual amount of 0.1N sodium hydroxide was titrated against N/10 hydrochloric acid solution using phenolphthalene as indicator



$$NaOH + HCl \longrightarrow NaCl + H_2O$$

Result

The volume of hydrochloric acid was measured through the end point when pink colour turned to colourless. The amount of cardondioxide was calculated by using the following formula:

mg carbondioxide = V x N x 22

V= volume of hydrochloric acid required to titrate residual sodium hydroxide

N= normality of hydrochloric acid

If volume of hydrochloric acid used is 50 ml and N is 1/10, the amount of cardondioxide evolved will be 50x1/10x22+110mg.

APPENDIX 20

DERMINATION OF GERMINATION INDEX

(Mathur *et al.,* 1993)

1. The germination index was determined by placing a layer of sample in the respective proportion in a petriplate and covering it with a filter paper.

2. Water was added until the filter paper was completely submerged and the seeds were placed on the filter paper.
3. Number of seeds germinating was measured after incubating the covered petri dishes in the dark at 28° C for 4 days.

Germination index was calculated using the formula

$$\text{Germination percentage} = \frac{\text{Number of seeds germinated}}{\text{Number of seeds sown}} \times 100$$

APPENDIX 21

DERMINATION OF ROOT AND SHOOT LENGTHS

The plants were uprooted washed with care in running tap water to remove adhering particles. Care was taken to ensure that the root system was not damaged. The plants were then gently blotted between filter paper folds to remove water droplets. Root lengths were measured from the ground level to the tip of the root and expressed in centimeters.

Shoot lengths of the plants were measured from the ground level to the shoot tops and expressed in centimeters.

APPENDIX 22

DERMINATION OF FRESH AND DRY WEIGHTS OF ROOT AND SHOOT
(Ali *et al.,* 2007)

After measuring the root and shoot lengths, the plants were weighed and the fresh weights were expressed in grams per plant.

The root and shoots were dried in an oven at 70° C for 24 hours and weighed. The weights were recorded as grams per plant.

APPENDIX 23

DERMINATION OF SOLUBLE SUGAR
(Mahadevan and Sridar, 1996)

The amount of total soluble sugars present in the plant extracts can be estimated by anthrone and phenol –sulphuric acid reagents. The sample should not be hydrolysed.

Principle

Anthrone, 10 keto-9, 10-dihydroanthracene, a reduction product of anthroquinone, reacts by condensing with carbohydrate furfural derivative to produce a green colour in a dilute solution and blue colour in a concentrated solution.

Reagents

1. 2 g of anthrone was dissolved in 1litre of concentrated sulphuric acid
2. 80 % ethyl alcohol

Plant extract preparation

1. The plant tissue (1g) was cut into pieces of 1-2cm; immediately and plugged in 5-10 ml of boiling ethyl alcohol and allowed to boiled for 5-10 minutes.
2. About 5 to 10 ml of alcohol was used for every gram of tissue. The extraction should be done on top of a steam bath or hot plate.
3. The extract was cooled and the tissues were crushed thoroughly in a blend for 5-10 minutes. It was passed through two layers of cheese cloth and reextrated the ground tissue reextracted for 3 minutes in boiling 80 % ethyl alcohol.
4. The second extraction ensured complete removal of alcohol soluble substances. Cooled and passed through cheese cloth.
5. The extracts were pooled and filtered through Whatman No.41 filter paper. The volume was raised with 80 % alcohol or reduced by evaporating to get a volume of 5-10 ml of the extract for every gram of tissue used.
6. To estimate reducing and non-reducing sugars, the alcohol in the extract was removed by evaporation and the aqueous fraction used for the analysis.

Method

1. Different aliquots of the extracts were pipetted into the test tubes. To each tube 4 ml of anthrone reagent was added along the sides of the tube.
2. A glass marble was placed to prevent loss of water by evaporation. The tubes were placed in a boiling water bath for 10 minutes.
3. The tubes were removed and cooled to room temperature.
4. The colour obtained was measured at 625 nm colorimetrically.

5. The amount of sugar present in the extract was calculated by using a standard graph prepared from glucose.

Comment

Alcohol interferes with colour development in the anthrone sugar reaction. Remove the alcohol by evaporation; when alcohol extract is used. To remove the plant pigments which interfere with the colour development pass the leaf extracts after diluting it with water to reduce the alcohol content to less than 50% through a column (1x10cm) of magnesium oxide.

APPENDIX 24

DERMINATION OF PROTEIN

(Lowry *et al.,* 1951)

Principle

The blue colour developed by the reduction of the phosphomolybdic – phosphotungstic acid 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 tartarate are measured by the Lowry's method.

Reagents

1. 2 % sodium carbonate in 0.1 N sodium hydroxide (Reagent A)
2. 0.5 % copper sulphate (CuSO4.5H2O) in 1% potassium sodium tartarate (Reagent B)
3. Alkaline copper solution: 50 ml of A and 1 ml of B prior were mixed (Reagent C).
4. Folin-Ciocaltteau Reagent (Reagent D) A mixture consisting of 100 g sodium tungstate (Na_2 $WoO4.2H_2O$), 25 g sodium molybdate (Na_2MoO_4. $2H_2O$), 700 ml water, 50 ml of 85 % phosphoric acid and 100 ml concentrated hydrochloric acid were refluxed gently for 10 hours in a 1.5litre flask. To this 150 g lithium sulphate, 50 ml water and few drops of bromine water was added. The mixture was boiled for 15 minutes without condenser to remove excess bromine. It was cooled and

diluted to 1litre and filtered. Care was taken to ensure that the reagent did not have greenish tint. The concentration is determined by titrating against 1N sodium hydroxide to a phenolphthalein end point.

5. Protein solution (stock standard): 50 mg of bovine serum albumin was weighed and dissolved in distilled water. Made the volume to 50 ml in a standard flask.

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

Procedure

Extraction of protein from sample

Extraction was usually carried out with buffers used for enzyme assay. About 500 mg of the sample was weighed and ground well with a mortar and pestle in 5-10 ml of the buffer. Centrifuged and used the supernatant for protein estimation as follows.

Procedure

1. 0.2, 0.4, 0.6, 0.8, and 1 ml of the working standard was pipetted into a series of test tubes.
2. 0.1 and 0.2 ml of the sample extract was pipetted into two other test tubes.
3. The volume was made to 1 ml with distilled water. A tube with 1ml of water served as the blank.
4. 5 ml of reagent C was added to each tube including the blank. The conents were mixed well and allowed to stand for 10 minutes.
5. Then 0.5 ml of reagent D was added, mixed well and incubated at room temperature in the dark for 30 minutes.
6. The blue colour developed was read at 660nm.
7. A standard graph was drawn to calculate the amount of protein in the sample.

Calculation

The amount of protein was expressed in 1g of sample

APPENDIX 25

ESTIMATION OF TOTAL FREE AMINO ACIDS

(Sadasivam and Manickam, 2008)

Principle

Ninhydrin, a powerful oxidizing agent, decarboxylates the alpha –amino acids and yields an intensely coloured bluish purple product which is colourimetrically measured at 570nm.

Reagents

1. Ninhydrin: 0.8 g stannous chloride was dissolved in 500 ml of 0.2 M citrate buffer (pH 5.0). this solution was added to 20 g of ninhydrin in 500 ml of methyl cellosolve (2 methoxyethanol)
2. 0.2 M citrate buffer pH 5.0
3. Diluent Solvent: n-propanol was dissolved with equal volume of water.

Procedure

Extraction of Amino acids

500 mg of the plant sample was weighed and crushed in a mortar and pestle with a small quantity of acid –washed sand. To this homogenate, 5 to 10 ml of 80% ethanol was added, filtered, centrifuged and the filtrate / supernatant was saved. The extraction with the residue was repeated twice to pool all the supernatants. The volume was reduced by evaporation and the extract was used for the quantitative estimation of total free amino acids. If the tissue was tough, boiling 80% ethanol was used for extraction.

Estimation

1. 0.1 ml of extract was added to 1 ml of ninhydrin solution
2. The volume was made to 2 ml with distilled water
3. The tube was heated in a boiling water bath for 20 minutes
4. After 15 minutes the intensity of the purple colour was read against a reagent blank in a colorimeter at 570 nm.
5. The reagent blank was prepared as above by taking 0.1 ml of 80% ethanol instead of the extract.

Standard

50 mg of leucine was dissolved in 50 ml of distilled water in a volumetric flask. 10 ml of this stock standard was diluted to 100 ml in another volumetric flask for working standard solution. A series of volumes from 0.1 to 1 ml of this standard solution gives a concentration range 10 µg. It was proceeded as for the sample and the colour was read.

Calculation

A standard curve was drawn using absorbance versus concentration. The concentration of the total amino acids in the sample was expressed as percentage equivalence of leucine.