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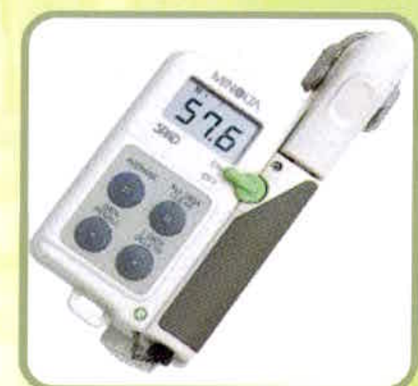
PRACTICAL MANUAL
ON
PRINCIPLES OF PLANT PHYSIOLOGY
(PP 501)



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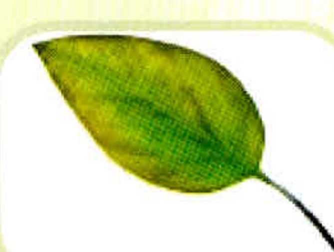
Chhattisgarh Samvad

NUTRIENT DEFICIENCY SYMPTOMS

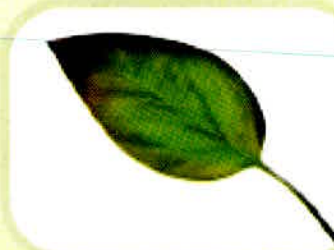
Nitrogen

Phosphorus


Potassium

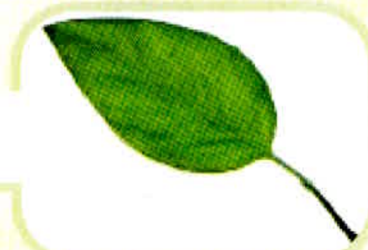
Calcium

Sulphur


Magnesium

NUTRIENT DEFICIENCY SYMPTOMS

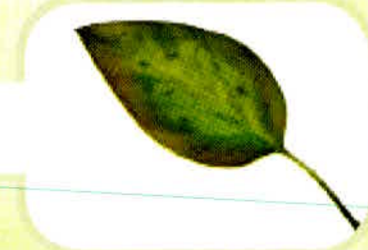
Iron


Manganese

Zinc

Boron

Copper


Molybdenum

PRACTICAL MANUAL

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Foreword

The discipline of Plant Physiology is one of the most pioneer branch of agricultural science. Several of the physiological tools and traits are commonly used in research in field of all cognate disciplines in improving crop productivity. The teaching in agricultural science need to be more practically oriented and students should be well aware of basic practical concept in translating the knowledge into action. In view of this, the publication of Practical Manual on Principles of Plant Physiology by Shri V.B. Kuruwanshi, Assistant Professor and Dr. Arti Guhey, Professor &Head, Department of Plant Physiology at College of Agriculture, IGKV, Raipur (C.G.) is timely efforts to help the students.

The description have been classified in a point wise, serial order and efforts have been made to collect latest information from several standard books to include all important points and details in a concise and lucid way, so that slight practice on the parts of the students will considerably add to their comprehension. This handy manual is very useful for Undergraduate and Post graduate course as well as young teachers in the field of Plant Physiology.

(S.K. Patil)

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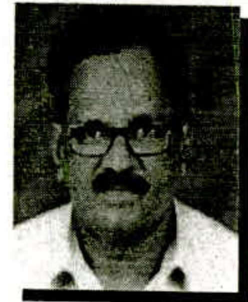

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MESSAGE

New information keeps on expanding and enriching every sphere of knowledge. Plant Physiology is more like a rule of this dictum. The teaching in Agriculture and Plant Sciences need to be more practically orientated for researchers and students. However, no standard method or techniques are being used by them due to compendium on the techniques. In view of this, a Practical Manual on Principles of Plant Physiology has been planned and make available the standard methods for Undergraduate and Post graduate students. This manual provide basic information about various physiological process, methods and techniques of Plant Physiology. The methods have been described in a simple wayjust like **"DO IT YOURSELF"** book.

I compliment Shri V.B. Kuruwanshi, Assistant Professor and Dr. Arti Guhey, Professor and Head, Department of Plant Physiology for their efforts for present manual. I am optimistic that Undergraduate, Post graduate and young teachers shall find this manual useful as an overview of the basic and applied aspects of Plant Physiology.

(S.S.Rao)

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PRACTICAL NO. 01

Object: Measurement of Soil Water Status by Pressure Plate Apparatus and Neutron Probe

The knowledge of soil water is essential not only to avoid water stress to crop plants but also to manage efficiently for higher water use efficiency. Soil water or soil moisture content is measured by pressure plate apparatus **(Fig 1.1)** and neutron probe or neutron scattering technique **(Fig 1.2)**.

(a) Pressure plate apparatus

Principle

Soil water potential is usually measured by pressure plate apparatus. It is consists of ceramic pressure plate or membranes of high air entry values contained in airtight metallic chambers strong enough to withstand high pressure of 15 bars or more. The apparatus enables development of soil moisture characteristic curves over a wide range of matric potential.

Procedure

The porous plates are first saturated and then soil samples are placed on these plates. Soil samples are saturated with water and transferred to the metallic chambers. The chamber is closed with special wrenches to tighten the nuts and bolts with required torque for sealing it. Pressure is applied from a compressor and maintained at a desired level. It should be ensured that there is no leakage from the chamber. Water starts to flow out from saturated soil sample through outlet and continues to trickle till equilibrium against the applied pressure is achieved. Soil samples are taken out and oven dried to constant weight for determining moisture content on weight basis. Moisture content is determined against pressure values varying from -0.1 to -15 bars. The values of moisture content so obtained at a given applied pressure are used to construct soil moisture characteristics curves.

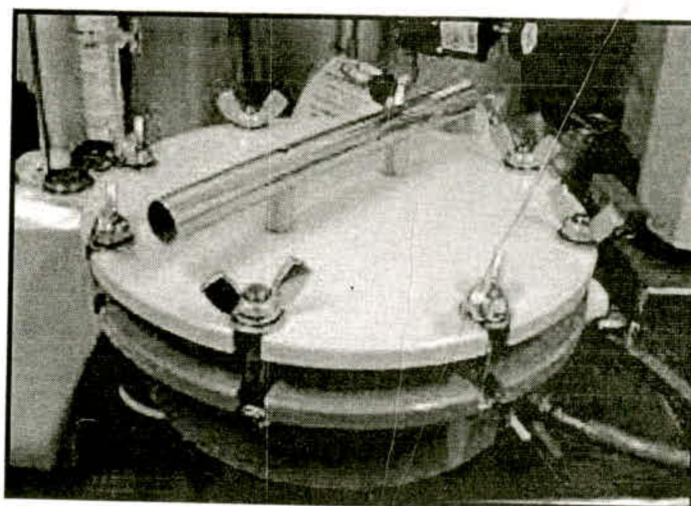


Fig 1.1: Pressure plate apparatus

(b) Neutron probe or Neutron scattering method

Principle

Fast neutrons emitted by a mixture of a stable element and a radioactive source, are slowed down by repeated collisions with nuclei of the surrounding elements and brought to the same thermal level as atoms of other substances. The neutrons lose maximum amount of energy when they strike an atom having almost the same mass as that of a neutron (Unit atomic weight). Hydrogen atoms are similar in mass to neutrons and thus contribute the maximum in thermalising them. This forms the basis of monitoring the soil water content using a neutron moisture meter assuming that the H atoms are part of the water molecules.

Components and working

Neutron moisture meter consists of a probe consisting of a detector and source that is lowered into the soil inside an aluminum access tube. The radioactive source is a mixture of a radioactive element and a stable element and their interaction supplies a continuous stream of fast neutrons. In an Americium/Beryllium source, Americium is the radioactive component while Beryllium is the stable element. Some of the thermalized neutrons (fast neutrons slowed down by repeated collisions) enter the detector, which is placed just behind the source. The detector consists of a metallic tube containing Boron tri-fluoride (BF_3) gas enriched with Boron. The fast neutrons react with the Boron and Alpha particles are produced by this reaction. The detector is able to register thermal neutrons only. The alpha particles thus produced in the detector cause ionization which generates electrical pulses. This pulse is amplified by a pre-amplifier housed in the probe itself and then transmitted to the scalar. The scalar counts the number of pulses (or neutrons) in a pre-set time interval.

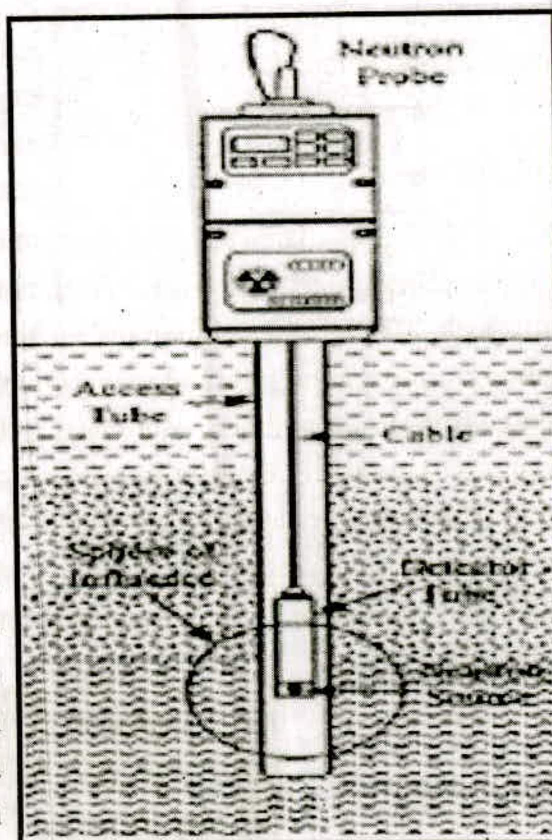


Fig 1.2: Neutron probe

Material required: Neutron moisture meter, aluminum access tubes, balance, oven, aluminum boxes and screw auger/tube auger.

Calibration

Initially, standard counts are taken with the probe inside the field. The shield acts as a standard having moisture 50 per cent by weight. Counts are generally taken for 1 minute intervals

but for precision, they should be taken for greater intervals. Observations are taken successively at 30 cm intervals after lowering the probe in the access tube. Count ratio is then computed by dividing the observed counts (Nm) by the standard counts (Ns). The Calibration curve is obtained by taking series of counts and soil samples for gravimetric estimation simultaneously at different depths.

Procedure

1. Install the aluminum access tube of specific diameter with closed bottom, up to the crop rooting depth. For this, a hole of diameter equal to outer diameter of the access tube is made in the field. The access tube is inserted in it, keeping- 20- 30 cm above the ground. Generally a 2-metre length access tube is installed. The gap between outer circumference of the tube and inner wall of the hole is filled with soil slurry in order to ensure proper contact of the access tube with the field soil. Cover the opening of the tube with a rubber stopper.

2. Before taking actual observation for the soil moisture, take standard count (Ns) by placing the neutron moisture meter on the top of the open access tube, keeping the instrument in ON position and timer at 4 minutes/calibration mark.

3. Lower down the probe into the access tube upto the desired soil depth. Put the instrument on measurement mode and the timer at the desired option of 4 minutes or 1 minute or 30 seconds. After the desired time record the measured count (Nm). Continue the same process to measure the count at lower depths.

4. Find out soil moisture against the count ratio (Nm/Ns) from the calibration curve prepared for this soil by measuring various count ratios and corresponding water content values at different soil depths. Obtain directly the soil moisture from the instrument in case there is built in microprocessor in the unit. * Standard counts are the average of the counts taken before and after the observations

Observations (Standard count*) =

Depth (cm)	Observed Counts (Nm)	Count ratio (Nm/Ns)	Soil water content (%)

PRACTICAL NO. 02

Object: Measurement of Relative Water Content (RWC) and Water Saturation Deficit (WSD) of leaf

Principle

Plant leaves generally have lower (more negative) water potential than pure water; hence, they osmotically absorb water and become turgid. A measure of this property is the Relative Water Content (RWC) which expresses the leaf water content (%) of the turgid leaf water content. It is calculated by the formula:

$$\text{RWC (\%)} \quad \frac{\text{Fresh weight (g) - Oven dry weight (g)}}{\text{Turgid weight (g) - Oven dry weight (g)}} \times 100$$

As similar parameter is the Water Saturation Deficit (WSD) that is computed by the formula:

$$\text{WSD (\%)} = \frac{\text{Turgid weight (g) - Fresh weight (g)}}{\text{Turgid weight (g) - Oven dry weight (g)}} \times 100$$

The water is absorbed till the existence of a Water Saturation Deficit (WSD) (i.e. till the tissue becomes fully turgid and stops absorbing water) so that RWC + WSD = 100% or WSD = 100-RWC (Kramer, 1983).

Material required

Leaf tissue, deioniesed water, Petri dishes, balance etc.

Procedure

Excise the leaf tissue (or any other tissue) which RWC is to be determined. Weigh the tissue and now dip the tissue in deionised water in covered Petri dishes or any other suitable container for 4-5 hrs. at room temperature till the tissue is fully saturated in a perfectly humid environment. Remove the tissue, blot it to remove surface water and weigh to get the turgid weight. Now dry the tissue to a constant weight in an oven at 65-70^{0}C for 72 hrs. and weigh it to record the dry weight. Determine the RWC from the recorded parameters **(Table.2.1)** as described as above:

Table 2.1: Calculation of Relative Water Content (RWC)

Tissue	Fresh weight (g)	Turgid wt. (g)	Dry weight (g)	RWC %
1.				
2.				
3.				

PRACTICAL NO. 03

Object: Measurement of Water Potential of Plant Tissue by Chardakov's Method

This method is developed by Chardakov's a Russian scientist, can be used in the laboratory as well as the field and does not required any sophisticated instrumentation.

Principle

Concentration of the solution in which a plant tissue is immersed changes (increases or decreases), depending upon the water potential of the tissue and the solution. If the solution is hypertonic (with lower Ψw) the tissue losses water and the solution becomes dilute. If the solution is hypotonic (with higher Ψw) the tissue absorbs water and solution becomes concentrated. If the solution is isotonic (with lower Ψw equal to the tissue sap), the tissue neither losses nor absorbs water and the solution concentration does not change. Thus the Ψw of this solution gives the Ψw of the tissue.

Material required

Leaves or any other tissue of the plant to be tested, test tubes, sucrose or polyethylene glycol (PEG) 6000 solution, methylene blue, dropper.

Procedure

Prepare graded concentrations of sucrose or PEG 6000 of different molalities *i.e.,* 0.05, 0.1, 0.15, 0.2, 0.25, 0.3 **(Fig 3.1)**. Take these solutions in two test tubes each. Colour one set of each solution by dissolving a small crystal of methylene blue in it (control solution). This does not change the Ψw of the control solution than to its non-coloured counterpart (test solution). Immerse tissue samples (approximately equal size) in each colourless test solution test tube. Allow the tissue to come into equilibrium with the solution for about 15-20 min. at room temperature. Remove the tissue from the test tubes. Put a drop of the corresponding coloured control solution in the colourless test solution in the center. Record the movement of the drop *i.e.,* whether it rises up, diffuses or sinks.

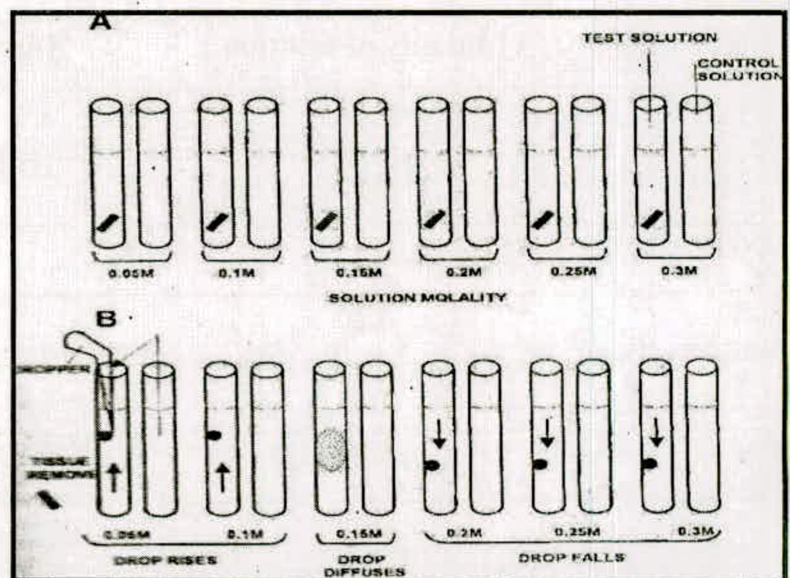


Fig 3.1: Measurement of water potential of plant tissue by Chardakov's method

Interpretation of results

If the control solution drop rises up the test solution, in which the tissue was incubated has become dense due to absorption of water by the tissue. If the drop sinks test solution has become less dense, having osmotically absorbed water from the tissue. If the drop diffuses evenly into the test solution without rising or sinking, then no change in the concentration has occurred and the water potential of the solution equals to that of the tissue. Chardakov's method is very simple and fairly sensitive for determination of plant tissues even in the field.

Precautions

1. To avoid evaporative losses, the test tissue should be immediately dipped in the graded solutions and solutions in the test tubes should be kept covered with glass marbles.
2. Tissue size does not affect the end result, but it should be of nearly uniform size/thickness to allow uniform equilibration time.
3. Allow sufficient time for tissue to equilibrate with the test solution. For non-waxy leaves 4-5 hrs time is sufficient.
4. Put the drop of control solution into test solution very gently.

The movement of control solution drop in test solution should be recorded in following **(Table 3.1)**.

Table 3.1: The movement of control solution drops in test solution.

S.N.	Molality of solution	Direction of drop movement*
1.	0.05	
2.	0.10	
3.	0.15	
4.	0.20	
5.	0.25	
6.	0.30	
7.	0.35	

*** Rises up, diffuses, sinks**

PRACTICAL NO. 04

Object: Measurement of Leaf Water Potential by Pressure Bomb and Psychrometer Method

The potential energy, per unit mass of water with reference to pure water is called as water potential. There are two main methods of measuring water potential:

(a) Pressure bomb or pressure chamber

Principle

The pressure applied to detached leaf to return the water interface, where it was before detachment, is equal and opposite to the tension in the xylem of the intact plant because the osmotic potential of the xylem sap is usually less than 0.02 Mpa, the hydrostatic pressure in the xylem is equal to the water potential.

Procedure

Leaves, after excision at petiole are put into butter paper bags. All such bags are closed in a polythene envelope. The envelope, after wrapping carefully is kept in a thermocol ice box containing ice cubes. This precaution is essential to prevent any further decline in leaf water potential due to desiccation. A sample leaf is inserted in a Pressure Chamber apparatus with petiole protruding out from the air tight gasket. Compressed, dry nitrogen gas is passed into the chamber slowly but constantly through a flow regulator until the xylem sap oozes out at the cut end of the petiole. Generally we use a magnifying lens to visualize the liquid drop at the cut end more clearly. As soon as oozing out of the xylem sap is noticed, the compressed nitrogen gas from the chamber is released immediately and the value of water potential is recorded (Fig 4.1). This instrument measures water potential in bars. However, bar may be converted in Pascals as follows:

1 Bar= 105 Pascal= 0.1 Megapascal

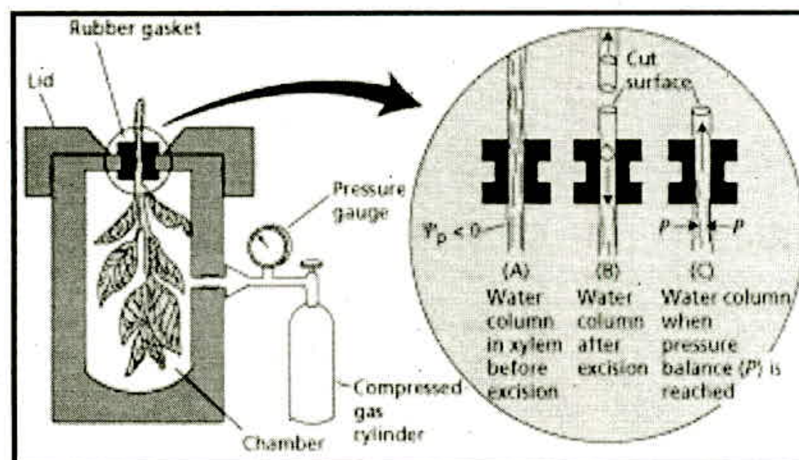


Fig 4.1: Pressure chamber with cylinder

(b) Psychrometer or vapour pressure method

Psychrometer can be used to measure the water potential of both excised and intact plant tissue. Moreover, the method can be used to measure the ?s of solutions.

Principle

The Psychrometry (Psychro- Greek word psychein, 'to cool') is based on the fact that the vapour pressure of water is lowered as its water potential is reduced. Psychrometer measure the water vapour pressure of a solution or plant sample on the basis of principles that evaporation of water from a surface cools the surface.

Procedure

A leaf disc is removed from the leaf and equilibrated in the chamber for approximately 20 minutes. The relative humidity of the equilibrated chamber is determined, and the chamber temperature and dry bulb minus wet bulb temperature are recorded. The reading is corrected to 25°C, and the water potential determined from a calibration chart. A calibration chart is needed for each psychrometer chamber and is usually compiled by the taking of measurements of distilled water and 1 molal NaCl. Each reading is corrected to 25°C, and the results are plotted as the reading in microvolts on the abscissa and water potential on the ordinate. For example, distilled water equals 0 water potential and 1 molal NaCl equals - 46.4 bars at 25°C (Fig 4.2). A major difficulty with this approach is the extreme sensitivity of the measurement to temperature fluctuations. For example, a change in temperature of 0.01°C corresponds to a change in water potential of about 0.1 MPa. Thus, psychrometers must be operated under constant temperature condition. For above reason, the method is used primarily in laboratory.

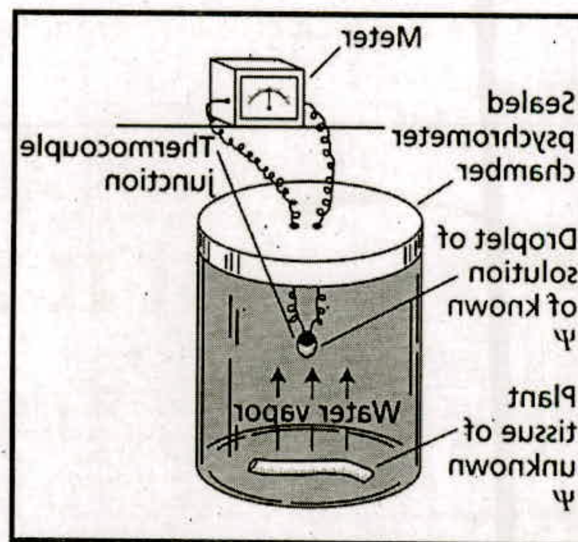


Fig 4.2: Psychrometer

PRACTICAL NO. 05

Object: Measurement of Transpiration Rate.

Stephan hales (1727) in his *vegetable staticks* first describe a method of demonstration transpiration in plants. The leaves of a well-watered plant were closed in transparent bell jar and water vapours condensing on the inner sides of the jar were seen. Hales devised a variety of method for measuring the rate of transpiration. Some of the methods are described here:

a) By weighing potted plants

A potted herbaceous plant is taken. It is well watered and the pot including soil surface is well covered with a waterproof material to prevent evaporation of soil. The pot is kept over a top pan balance and loss of weight over a definite time is noted **(Fig 5.1a)**.It will be reasonable to employ this method of transpiration measurement over short periods only. If the measurements are continued for a few days, the increase in plant weight may occur due to growth.

b) By Ganong's potometer

With Ganong's potometer (meaning drink measurer), the rate of water loss from a cut plant or shoot can be measured assuming that the rate of water absorption is equal to the rate of transpiration. There are several types of potometers. In one type, there is a water reservoir to which the plant or its parts are fitted **(Fig 5.2b)**. To this reservoir a glass capillary tube of known diameter is attached. A scale is also attached to the glass tube. An air bubble is introduced in the capillary tube and its movement is recorded with the help of scale. From the diameter of the tube, the amount water absorbed (hence amount transpired) can be calculated.

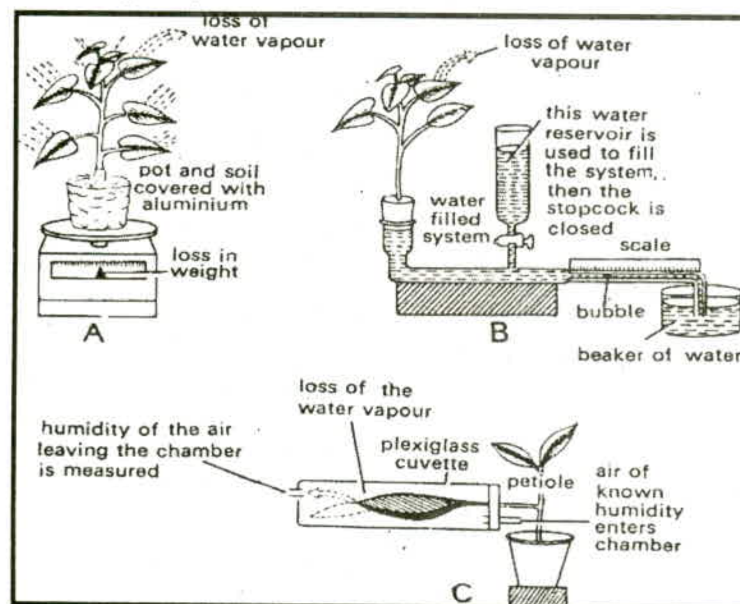


Fig 5.1 (a): By weighing a plotted plant (b) By a Ganong's potometer (c) By measuring humidity in the air

c) By measuring the humidity of air

This method can be used for measuring the rate of transpiration from single leaf. The leaf is enclosed in a Plexiglass (plastic glass) chamber and sealed. Air is passed through the chamber at a definite flow rate. The humidity of the air before entering the chamber and after leaving the chamber is measured **(Fig 5.1c)**. The amount of water transpired can be calculated by finding out the difference in humidity. Different methods are available for measuring the humidity of the air. In a simpler method, humidity is measured by collecting it through hygroscopic substances such as P_2O_5 or $CaCl_2$. The attached leaves are enclosed in the chamber which is also contains small tubes containing weighed amount of P_2O_5 or $CaCl_2$. In a control chamber, similar tubes are placed but no leaf is enclosed. The tubes are weighed after a definite time and the amount of water transpired by leaf is calculated.

PRACTICAL NO. 06

Object: Measurement of Leaf Resistance (or Stomatal Conductance) to Water Vapour with a Diffusion Porometer

Principle

A diffusive porometer measures diffusion of water vapor from the substomatal chambers through the stomata of an intact plant. The transient porometer which is one type of diffusive porometer, measures the time taken for a portion of a leaf enclosed in a cuvette to raise the humidity inside the cuvette over a fixed humidity interval. This interval can be converted to resistance by the use of an empirical calibration curve. Calibration involves replacing the leaf in the cuvette with a wet surface (usually water saturated filter paper) covered by a calibration plate with known resistance.

Material

The porometer **(Fig 6.1)** consists of

1) The leaf cuvette which is made up of a Vaisala Humicap capable of responding rapidly to changes in humidity, and two thermistors. One thermistor measures the temperature of the cuvette, the other measures leaf temperature.
2) The control console, which consists of a battery, a pump which circulates air through the cuvette, a container filled with desiccant which dries the water vapor from the air in the cuvette, a switch controlling humidity ranges, a timer, and a relative humidity/temperature gauge.
3) A calibration plate.

Procedure

1. Set the porometer function switch to the relative humidity setting (RH), open the cuvette and wave it in the air a few times. This will give you an idea of ambient relative humidity. Based on the reading obtained, select the proper humidity range for your measurements using the relatiye humidity range switch.
2. Next, you should calibrate the porometer using the calibration plate provided with the instrument. Wet a rectangular piece of filter paper with distilled water to the point of saturation, but not to the point of having water drip from the paper. The paper is placed beneath a layer of tape on one side of the calibration plate which has precisely drilled lines of holes. The number and size of these holes determine their resistance to water vapor diffusion. The cuvette is clamped over these holes, and the temperature of the cuvette, the

temperature of the calibration plate and the time it takes for humidity to change over a certain range is recorded for each series of holes. Plot the data on graph paper.

3. Then measure leaf resistance by clamping the cuvette gently onto the abaxial side of the leaf, for lower leaf resistance. The measurement interval begins when the relative humidity reaches a certain level inside the cuvette. As relative humidity rises within the cuvette, due to transpiration from the leaf, the instrument will start timing at a preset minimum relative humidity. When the relative humidity reaches a specified maximum, the time measurement stops. When this point is reached, the air pump starts which forces air through the dessication column and back to the cuvette, lowering humidity past the lower set point, at which point the pump turns off, and the humidification cycle starts again. As the pump dries the cuvette, the results of this measurement are displayed. In order to allow equilibration between the leaf and the cuvette, measurements should be repeated 3-5 cycles before the cuvette is removed from the leaf.

4. Take a drought stressed plant or a dark treated plant, and measure the leaf resistance as in step 3. Is there a difference between a light versus dark grown plant?

Fig 6.1: Diffusion Porometer

Precautions to observe

1. Avoid breathing directly on the leaf because high CO_2 in your breath may close the stomata.
2. Avoid touching or getting any water on the sensor. It is very sensitive to liquids and to oils on your hands. Moisture condensation may occur if you clamp the cuvette onto a leaf without having the pump activated, or if you have too much water on the filter paper used during calibration.

PRACTICAL NO. 07

Object: To study the Factors Regulating Guard Cell Movement and Stomatal Aperture

The stomatal aperture regulates gas exchange needed for photosynthesis and water loss by transpiration. In order to optimize photosynthesis and reduce water loss the aperture is regulated by the movement of two guard cells. The turgor pressure in leaf guard cells is used to open and close the stomatal opening. Environmental (e.g. light) and chemical signals cause ion movements that result in an increase of solutes in the guard cells. For example, light stimulates the plasma membrane H+-pumping ATPase, causing a change in the membrane potential which leads to the influx of K+. The increase in solute concentration causes water to follow. The turgor increase opens the stomata. These pressures can be very high; ranging upwards to 25 or 30 atm. During periods of water stress, stomatal apertures decrease and close to reduce water loss. The response to water stress is mediated in part by hormones, like ABA.

In this experiment, we test the effect of light and dark on stomatal opening and test the requirement for K+. The effect of a hormone abscisic acid, ABA, will also be tested.

Approach

Epidermal strips taken from leaves are exposed to various treatments, and observed under a microscope to determine the extent of stomatal closing or opening.

Procedure

a. What signals and ions alter guard cell movement? Why?

1. Prepare five petri plates with the following solutions:

    100 mM KCl, light

    100 mM KCl, dark

    200 mM mannitol, light

    100 mM choline Cl, light

    100 mM KCl, 10 μM ABA, light

2. Remove a healthy leaf by tearing the leaf parallel to the mid-vein. In the dark, peel the lower epidermis (guard cells are in the epidermal layer of cells) from the leaf using forceps. DO NOT ALLOW THE EPIDERMAL STRIP TO DRY. Place several strips into the solution.
3. Place plates in light for 1 hour, except for 'dark' treatment - put that one in a drawer.
4. Examine the light and dark treated sections under the microscope. Look for pairs of guard cells in a given area (Fig 7.1). Examine at least 20 pairs for each treatment. Note the number of stomates that are wide open, open, nearly closed or closed. Present results in a Table 7.1.

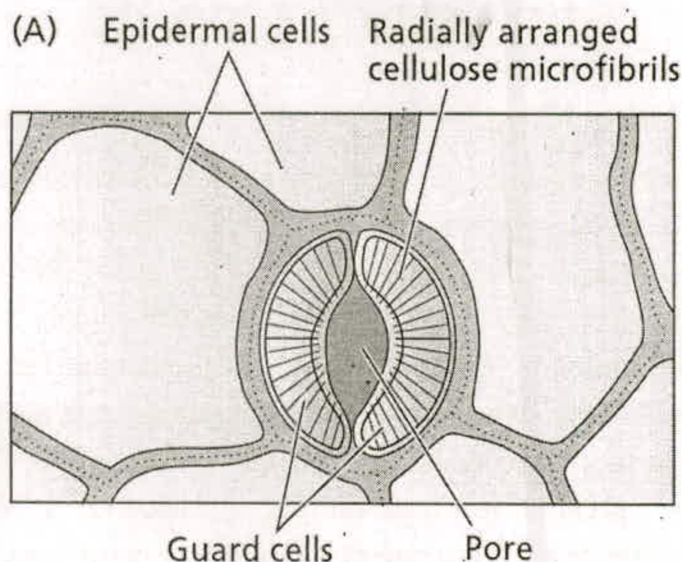


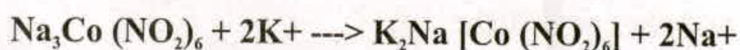
Fig 7.1: Stomata

Table 7.1: Observations

Treatment	Number of stomata			
	Wide Open	Open	Nearly closed	Closed
Dark, KCl				
Light, KCl				
Light, Mannitol				
Light, Choline Cl				
Light, ABA, KCl				

b. Visualizing K+ in cells: Histochemical Localization of K+

5. Place the epidermal peel (from 100 mM KCl solution in light and dark) in a cold 10% solution of sodium cobaltinitrite for 5-10 min. This reagent is used to reveal the levels of K+ in open and closed stomata by converting the soluble ion into an insoluble precipitate.



$$Na_3Co\ (NO_2)_6 + 2K^+ \dashrightarrow K_2Na\ [Co\ (NO_2)_6] + 2Na^+$$

6. Rinse the stained peel for 2-3 min in cold deionized water and mount the tissue in a drop of 5% glycerine. Observe under a microscope.

PRACTICAL NO. 08

Object: To study the Deficiency symptoms of Nutrients

All green plants are autotrophic and they are required from outside the supply of only inorganic substances. With the exceptions of C, H and O, all inorganic plant requirements are obtained directly or indirectly from soil. As the source of these inorganic requirements are minerals, the elements are known as mineral nutrients and the nutrition is called *mineral nutrition*.

Types of Essential Elements

1) **Macro elements (Macro nutrients):** Required by the plants comparatively large amount. C,H,O, N,P,K, (Primary) and Ca, S, Mg (Secondary).

2) **Micro elements (Micro nutrients or Trace elements):** Small quantity required by the plants. Fe, Mn, Zn, B, Cu, Mo, Cl

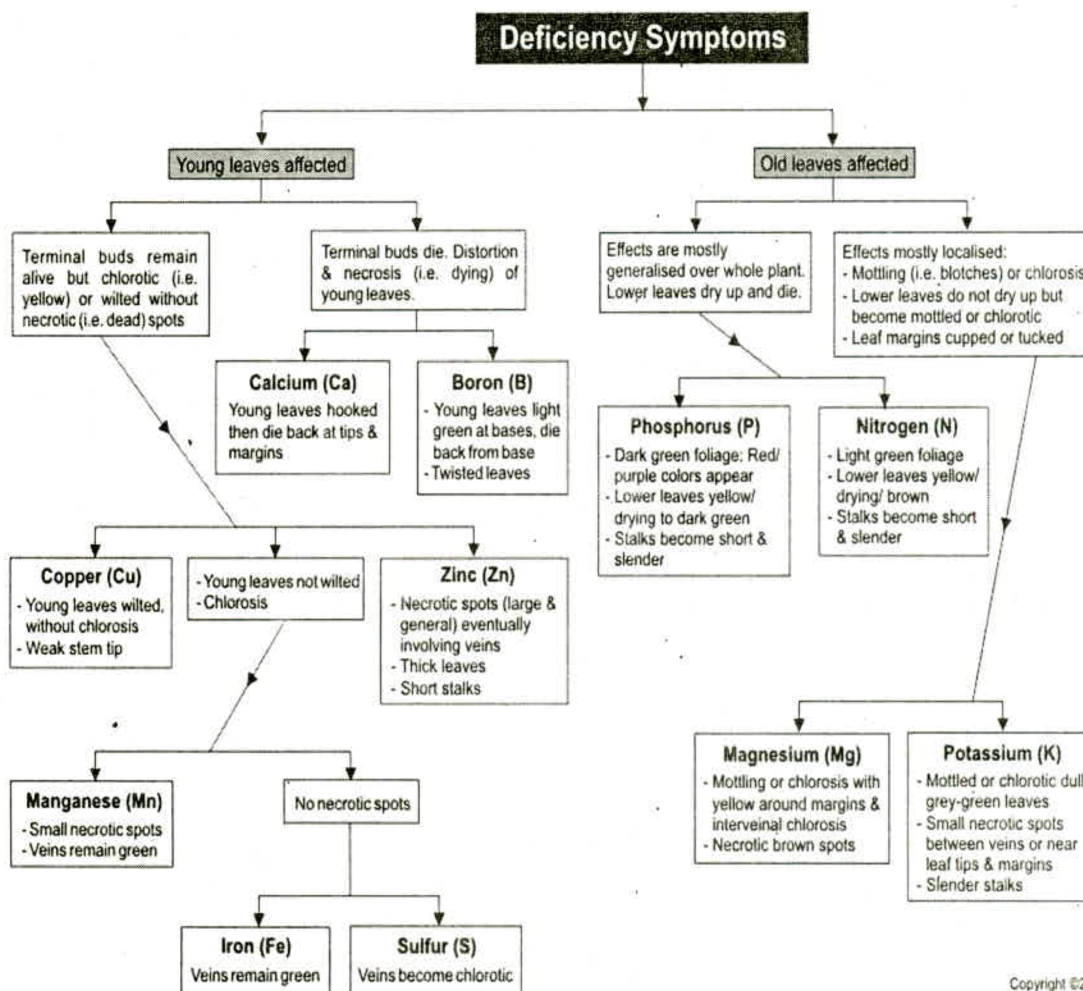


Fig 8.1: Diagnostic Flow Chart for Nutrients Deficiency Symptoms

PRACTICAL NO. 09

Object: To Study the Separation of Chlorophyll Content by Paper Chromatography

The chloroplast pigments (chlorophyll *a*, chlorophyll *b*, carotenes and xanthophylls) are situated on the thylakoid membrane of the chloroplasts. These pigments participate in the photochemical reactions of photosynthesis.

Material Required

Fresh spinach leaves or other fresh green leaves, 80 % acetone (v/v), carbon tetrachloride, petrolium ether, benzene, Mortar and pestle, filter paper, Buchner funnel, chromatography jar, chromatography paper, support rods, glass tubings drawn to a fine tubing, paper clips, hair dryer, ruler.

Procedure

Pigment extraction: Grind 500 mg of small cut pieces of fresh leaf material in 20 ml of 80 % acetone for about 5 min in a clean mortar. Carefully transfer the resulting green liquid to a Buchner funnel containing a layer of Whatman no. 1 filter paper. Filter the extract using suction.

Separation of pigments: Pour carbon tetrachloride or a mixture of petroleum ether and benzene (9:1, v/v) in a chromatographic jar to a depth of about 2 cm. Allow the internal atmosphere in the jar to equilibriate for few hours. Cut chromatography paper (Whatman no.1 paper) to a desired size and draw a pencil line about 2 cm away from the bottom. With the help of a glass tubing drawn to a fine tip spot two or three points about 3 cm apart from one another with pigment extract. Allow each pigment drop to dry completely before applying the next drops. Drying may be hastened by using a hair dryer. Repeat the application of drops until the marks are deep green. (The number of drops required will depend upon the concentration of pigment in the extract. Normally, 5-10 drops may be required). Hang the paper in the chromatography jar with the lower end dipping in the solvent. Close the jar. Remove the paper when the solvent has moved up to the top of the paper. Allow the paper to dry.

Results

Observe the separation of pigments on the paper and record the results as indicated in Table 9.1. Mark the spots with a pencil since the colours will fade away quickly. Calculate the R_f value of each pigment.

The compounds on the chromatogram can be identified on the basis of their diagnostic feature, the ratio of fronts (R_f) values. R_f is the ratio of the distance travelled by the substance to the distance travelled by the the solvent in a chromatogram:

$$R_f = \frac{\text{Distance from origin to pigment}}{\text{Distance from origin to solvent front}}$$

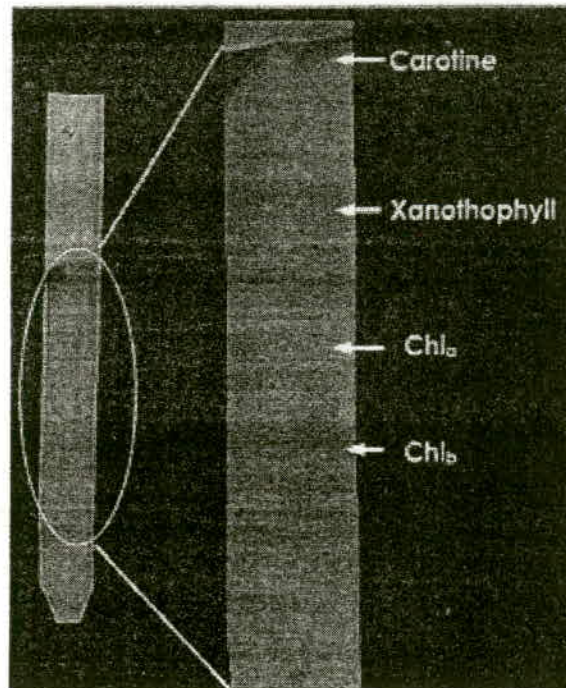


Fig 9.1: Paper Chromatography

Table 9.1: Identification of pigments alongwith their characterstics range of colours.

Spot/Band	Distance travelled	R_f value	Colour	Identification
Solvent front	9.1			
Band 1	9.0	0.989	Orange Yellow	Carotene
Band 2	1.7	0.187	Yellow	Xanthophyll
Band 3	0.9	0.099	Bluish green	Chlorophyll *a*
Band 4	0.4	0.044	Yellowish green	Chlorophyll *b*

PRACTICAL NO. 10

Object: Estimation of Chlorophyll Content

Chlorophyll can be regarded as true representation of health of green plants. This presumption is based on the face that chlorophyll is the most commonly and immensely influenced by biotic and abiotic stress conditions in addition to events like pathogenic invasion, pollution, mineral deficiencies as well as mineral toxicity. This is the reason that estimation of chlorophyll is considered to be one of the most important parameters in plant physiological studies.

(a) Acetone method

Chlorophyll is soluble in acetone. When the sample is macerated in acetone, chlorophyll gets dissolved in it. The optical density of the extract is measured at 663 and 645 nm wavelengths using a spectrophotometer or colorimeter because at these wavelengths, maximum absorption of chlorophyll 'a' and 'b' takes place, respectively. The amount of chlorophyll 'a', 'b' and total chlorophyll are determined using the formulae given by Arnon (1949).

Chlorophyll 'a' = [(12.7 x A663) - (2.69 x A645)] x V/1000 x W

Chlorophyll 'b' = [(22.9 x A645) - (4.68 x A663)] x V/1000 x W

Total Chlorophyll = [(8.02 x A663) + (20.2 x A645)] x V/1000 x W

The value, so obtained, are expressed as mg chlorophyll per gram fresh weight of sample, where;

A = Absorption at given wavelength,

V = Total volume of sample in extraction medium and

W = Weight of sample (g)

Material Required

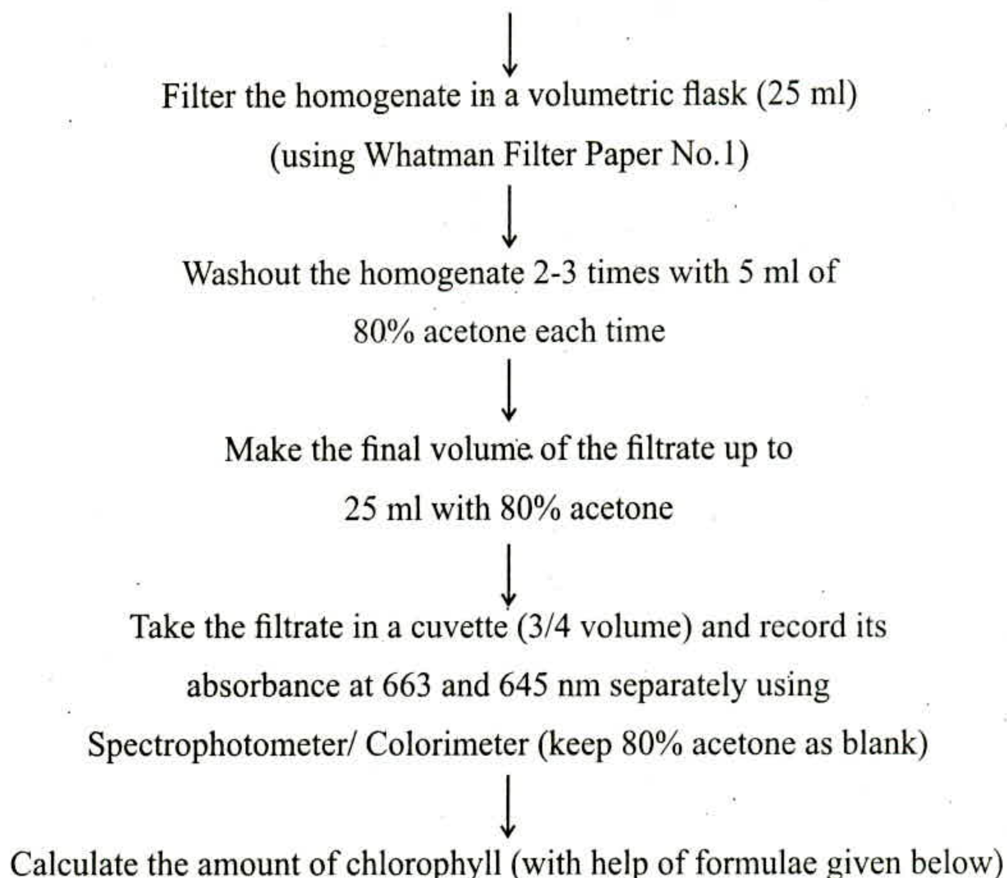
(i) Acetone (80%), (ii) Pestle and Mortar, (iii) Funnel, (iv) Whatman Filter Paper No.1, (v) Volumetric flasks (25 ml), (vi) Measuring cylinders (50, 100 ml), (vii) Pipettes, (viii) Test tubes, (ix) Spectrophotometer/Colorimeter, (x) Microbalance, (xi) Plant material.

Procedure

Take 200 mg freshly cut material

(leaf pieces devoid of mid rib)

Grind in a pestle with mortar (use 10 ml 80% acetone)



↓

Filter the homogenate in a volumetric flask (25 ml)
(using Whatman Filter Paper No.1)

↓

Washout the homogenate 2-3 times with 5 ml of
80% acetone each time

↓

Make the final volume of the filtrate up to
25 ml with 80% acetone

↓

Take the filtrate in a cuvette (3/4 volume) and record its
absorbance at 663 and 645 nm separately using
Spectrophotometer/ Colorimeter (keep 80% acetone as blank)

↓

Calculate the amount of chlorophyll (with help of formulae given below)

Precautions

(i) Extraction should be carried out at subdued light and low temperature because light influences pigments.

(ii) 2-3 washings are necessary to ensure maximum extraction of chlorophyll pigments.

(b) By SPAD or Chlorophyll Meter

It is a sample and non-destructive method of chlorophyll estimation. It can be used in the laboratory as well as in the field of standing crop **(Fig 10.1).**

Principle

SPAD (Soil and Plant Analytical Development) is a simple diagnostic tool. We get readings in terms of SPAD values that indicate relative chlorophyll content. It is based upon a unique linear relationship between SPAD and leaf area based nitrogen concentration in plants. However, it requires standardization/calibration for each crop because this relationship varies with crop growth stage and variety.

Fig 10.1 : SPAD or Chlorophyll meter

Procedure

(i) Switch on the instrument and let it warm up for about 10-15 minutes.

(ii) Calibrate the instrument for accuracy check using a special disc provided with the instrument.

(iii) As soon as the 'beep' sound is over, put a fully expanded leaf into it in such a way that only the leaf portion between the lamina and the mid-rib remains inside.

(iv) Note down the reading. To check a reading, take another reading on the same leaf.

(v) Use 'average' button to calculate the average of readings taken automatically.

(vi) Close until you hear 'beep'.

Precautions

(I) If you want to take reading of a detached leaf in the laboratory, care should be taken so that leaf does not wilt.

(ii) While working in the field, you should shield the leaf (sample) from direct sunlight.

PRACTICAL NO. 11

Object: To study the Oxygen Evolution during Photochemical Reaction

During photochemical reaction of photosynthesis, taking place in the chloroplast lamella system, electron flow from water to NADP. Simultaneously, water is oxidized and O_2 is released. This reaction is reaction is known as Hill reaction. The rate of production of NADP and ATP in photochemical reaction is directly associated with the oxidation of water the sole electron donor. So the photochemical efficiency can be measured by the rate of oxygen evolution.

Principle

Indigo carmine is blue in aqueous medium when it is in the oxidized state. When powerful reducing agents like sodium dithionate is added, the dye gets reduced to a colourless form. This reduced indigo carmine can be reoxidized by oxygen. On reoxidation, indigo carmine changes its color black to blue. The change in color of the dye to blue is therefore an indication of oxygen evolution.

Plant material

Fully expanded young leaves of sunflower or any other crop species.

Preparation of the stock solution

(i) Indigo carmine (indigo di sulphonate 0.01% w/v): Dissolve 10 mg of the dye in 100 ml of distilled water, (ii) Sodium dithionate (10%): Dissolve 10 g of sodium dithionate in 100 ml of distilled water. After preparing the solutions cover them tightly without exposing them to air.

Procedure

Take 10 ml test tubes with tight rubber stoppers and fill the test tubes with 0.01 per cent indigo carmine solution. Carefully add dithionate solution drop by drop until the blue indigo carmine is reduced to a colorless form. Place a few freshly harvested leaf bits in the solutions and seal the test tubes with a tight rubber stopper. Care must be taken to see that no bright light. Keep a set of test tubes in darkness to avoid photochemical reactions to serve as controls. Blue areas will appear around the leaf bits kept in bright light. Note down the time taken for the appearance of this blue areas around the leaf discs. The small amount of oxygen produced during photochemical reaction inside the leaf will reoxidize the reduced indigo carmine back to the blue color. The extent of blue color formed in the test tubes will be directly proportional to the amount of O_2 evolved.

PRACTICAL NO. 12

Object: Measurement of Photosynthetic Rate by IRGA

In all the gas exchange equipments designed to measure the photosynthetic rates, the Infra-Red Gas Analyzer (IRGA) is a major component and the primary role of this component is to measure the CO_2 concentration. The IRGA is very sensitive to detect even a change of 1 ppm of CO_2. A leaf or a plant is enclosed in an airtight chamber and the CO_2 fluxes are determined by measuring the CO_2 concentration changes in the chamber atmosphere (Fig 12.1).

Principle of IRGA

Infra-Red Gas Analyzers (IRGA) are used for the measurement of a wide range of Heteroatomic gas molecules including CO_2, H_2O, NH_3, CO, SO_2, N_2O, NO and gaseous hydrocarbons like CH_3. Heteroatomic molecules have characteristic absorption spectrum in the infrared region. Therefore, absorption of radiation by a specific Heteroatomic molecule is directly proportional to its concentration in an air sample.

The major absorption peak of CO_2 is at 4.25 µm with secondary peaks at 2.66, 2.77 and 14.99 µm. The only heteroatomic molecule normally present in the air with an absorption spectrum overlapping with that of CO_2 is water vapour. Both molecules absorb IR radiation in the 2.7 ?m range. However, this interference does not pose any serious problem at 4.25 µm range.

Measurement of photosynthesis

Two different kinds of photosynthesis systems (IRGA) are generally used for the measurement of photosynthetic rates *i.e.* closed system and open system (Fig 12.2).

(1) Closed system

In this system, a leaf is clamped in a leaf chamber and air is circulated around the leaf repeatedly. The decrease in Co_2 concentration over time is measured to determine the photosynthetic rate. The major disadvantage in this system is that the measurements of photosynthetic rates are done under constantly depleting CO_2 concentrations around the leaf. Further, since the leaves transpire simultaneously there will be a constant build up of humidity in the chamber. Increase in humidity alters the vapour pressure difference (VPD) between the leaf and its ambient air. Small changes in VPD have been shown to significantly alter the stomatal opening and hence the gas exchange rates. These disadvantage are overcome in the open system of measurements.

(2) Open system

Here the leaf is clamped in a chamber through which ambient air is passed continuously. The change in CO_2 concentration before the air enters the leaf chamber and in the air leaving the leaf chamber is determined to compute the photosynthetic rates. If photosynthetic rate is high, then the

air leaving the leaf chamber will have less CO_2 than the air entering it. The difference between the CO_2 concentrations at a given flow rate is determined to compute the photosynthetic rate.

The major advantage in the open system is that, the measurement of photosynthetic rates can be achieved at a constant CO_2 concentration. Further, by altering the flow rate of dry air into the leaf chamber, it is also possible to maintain a specific relative humidity in the chamber and hence the gas exchange parameters can be measured at a constant set of conditions. This renders the comparison more realistic and acceptable.

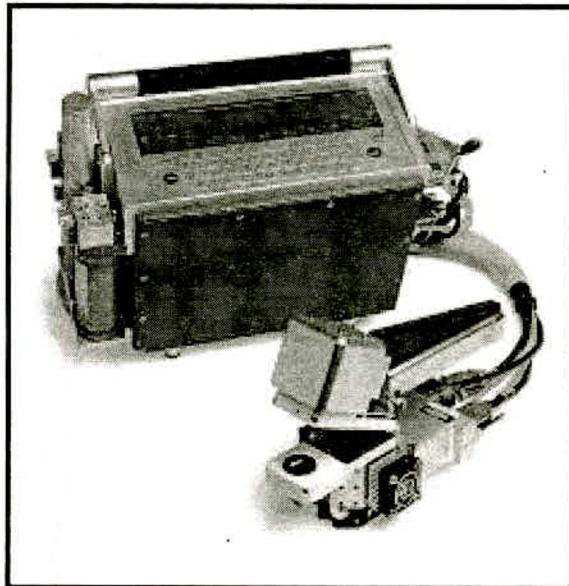


Fig 12.1: IRGA

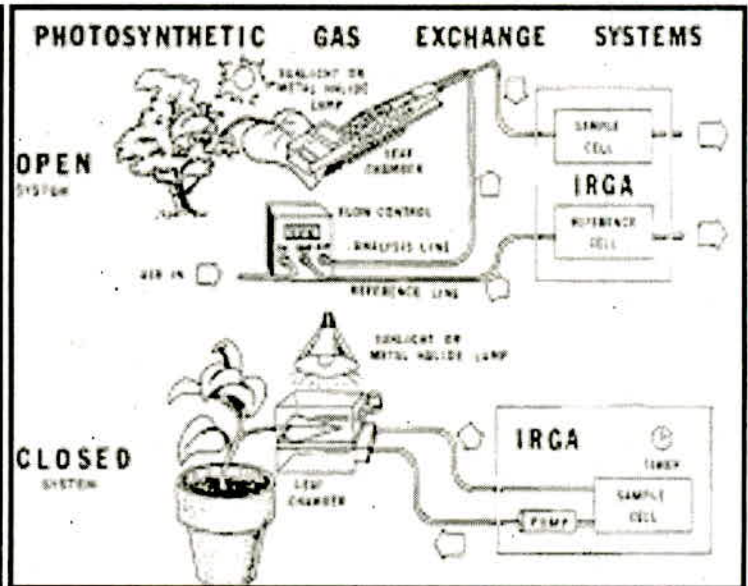


Fig 12.2: Closed System & Open System

PRACTICAL NO. 13

Object: Measurement of Respiration Rate

Respiration rate, irrespective of plant parts (*viz.* leaves, fruits or seeds), is considered to be an important parameter in plant physiological studies. Respiratory gas exchange is just reserve on opposite to that of photosynthesis and as the later is an energy building phenomenon where ATP is stored in the photosynthates produced; respiration involves breakdown of photosynthates for release of energy to be utilized for the performance of other activities of life. On the basis of respiration a climacteric fruit can well be distinguished from a non-climacteric one; as the former reveals a characteristic 'Climacteric Bust' in respiration, which is completely lacking in the later.

There are few instruments used for the measurement of respiration in plants viz.

(a) Infra-Red Gas Analyzer

It is generally used for the measurement of photosynthesis. However, it can also be used for measuring respiration provided:

1. The sample chamber (containing leaves) is covered with dark black cloths; so that no light reaches the leaf surface and photosynthesis may be avoided; and
2. Necessary tubing connections are made depending upon the 'closed' or 'open' type of measurement mode, as required.

Here, we measure the rate of evolution of CO_2, Which is taken to be the rate of respiration.

(b) Respirometer

Basically, it is an Infra-Red Gas Analyzer, however, its CO_2 measurement range varies from 0-50,000 ppm (µmol mol-1) or even higher. Hence, it can be conveniently used for the measurement of respiration of large amount of sample such as fruits of big size etc. Secondly, this instrument is equipped with an 'Auto Zero' device which allows for fast warm up, adaptation to changing ambient conditions and excellent stability for the CO_2 signal (Fig 13.1).

Principle

Gases with di-atomic molecules such as CO_2 strongly absorb photon in the infra-red range. For CO_2, one region of strong absorption is 4.26 microns. A source emitting strongly at this wavelength is a light bulb. If this is positioned at one end of a tube and at the other end is placed a sensor that is sensitive to photon at 4.26 micron, we have a simple infra red analyzer. As carbon dioxide is passed down the sample cell, it absorbs some of the infra-red radiation and the sensor reading decreases.

Respirometer is consists of the following parts: (1) Electronic housing with air supply fan (2)

Air supply chimney, (3) Sample chamber (4) Environmental Gas Monitor for CO_2 (EGM-4)

There are two gas ports on the top of the EGM-4; 'Gas in' and 'Gas out'. The sampling line is fitted to 'Gas in' and 'Gas out' line is left open to atmosphere to allow the sample air to exhaust without restriction (open system). For 'closed system', one gas line of the sample chamber is connected to the 'Gas in' port and the other to the 'Gas out' port. On the rear panel, there is an absorber column. It contains soda lime. When air passes through this column, it removes all of the CO_2 from the air stream. The 'Auto Zero' facility, built into the EGM-4, periodically switches the flow of the gas from the analyzer through this column to check the analyzer zero. This routine ensures long term stability and accuracy of the CO_2 analyzer. Soda lime turns green to brown when exhausted. It should be removed with fresh stock periodically. The EMG-4 has one connector used for transferring stored data to a PC or Laptop computer. It has a 2x16 character backlit LCD display. The keypad consists of 12 keys, each having specific function. The system is supplied with an AC adaptor/ charger for charging the internal 12V lead acid battery.

Assembly

The air supply chimney is pushed into the air supply fan and a 3-pin electrical connector (from the Mass Flow Meter) is connected to the Electronic Housing. The exhaust pipe is attached to the sample chamber and the later is pushed on to the port next to the air supply fan. The sample chamber is also connected with the Electronic Housing using a 6-pin connector. The electronic housing is finally connected with the EGM-4.

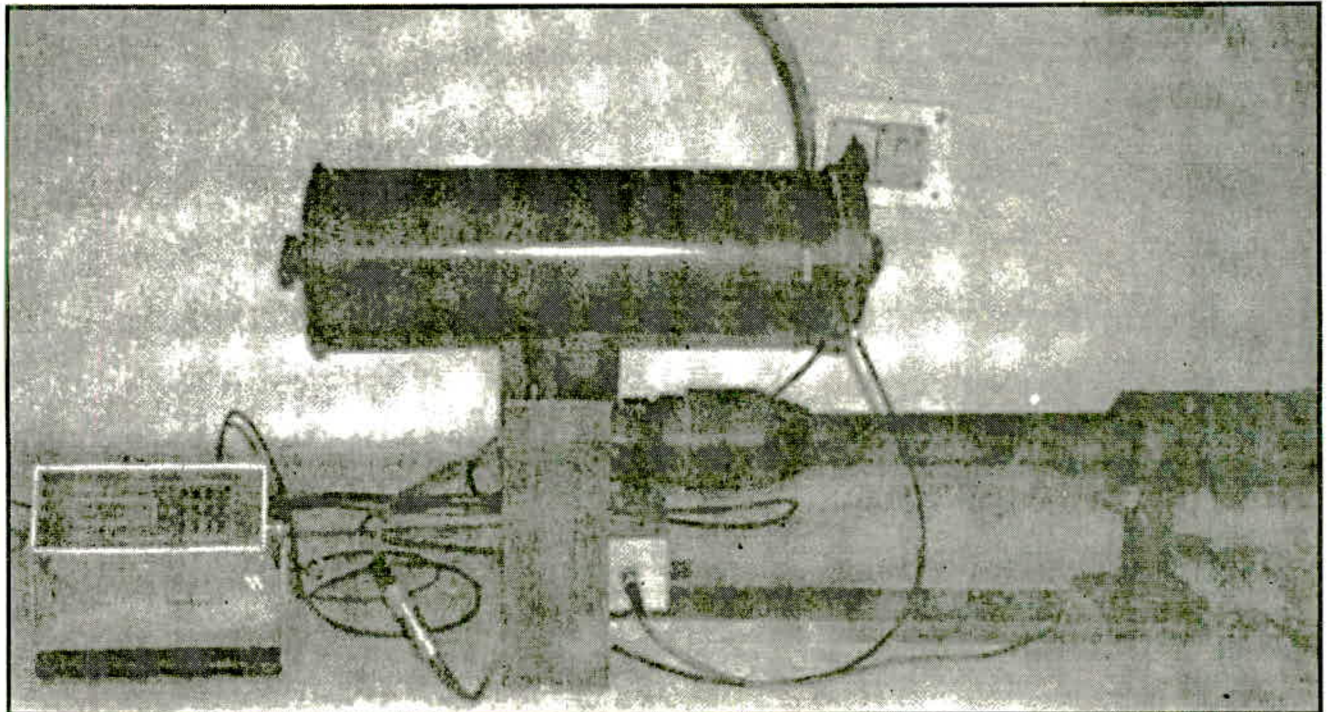
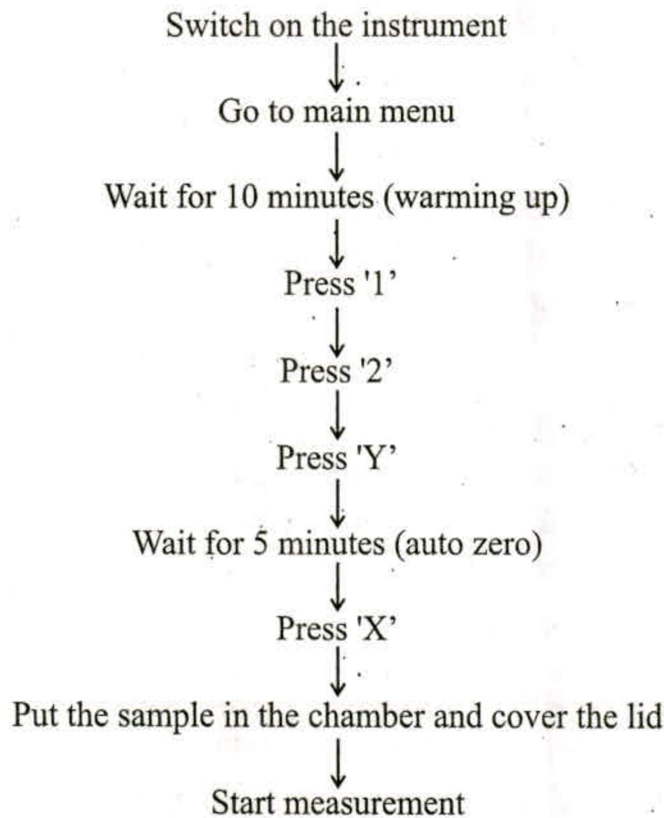


Fig 13.1: Respirometer

Operation

Air is pushed through the sample chamber by the air supply fan and flow rate is controlled by the mass flow meter. Rotating the flow control knob on the electronic housing, the flow rate in the chamber is adjusted.

The air flow rate should be adjusted to give 20-30 ppm differentials. Thereafter.



The abbreviated form of digital output appears as follows:

Rnnnnn Dnnnn Tnn

A+/- nnnn Fnnnnn

Where,

R = Reference Co_2

D = Differential Co_2

T = Chamber temperature

A = Assimilation rate

F = Flow rate (ml/min.)

Measurement is automatically corrected for temperature and pressure.

Results are given in µmol m-2 s-1 for Co_2

To convert it into g CO_2 m-2 h-1, the values should be multiplied by 0.1584.

PRACTICAL NO. 14

Object: Estimation of Reducing, Non-Reducing and Total Sugars

The most abundantly and commonly available compounds in the plant kingdom are carbohydrates. They comprise saccharides viz.

Monosaccharides - Glucose, Fructose etc.

Disaccharides - Sucrose, Lactose, Maltose etc.

Polysaccharides - Starch, Cellulose etc.

Cellulose is the major component of plant cell wall.

The active groups in carbohydrates are aldehyde (-CHO) and ketone (=CO). In addition, they contain hydroxyl groups as well. The chemical properties of saccharides vary depending upon the number of hydroxyl groups and the presence or absence of (-CHO) or (=CO) groups.

I. Estimation of Reducing Sugars

Principle

The reducing property of sugars arise out of the presence of a potential aldehyde or keto group. Nelson (1944) and Somogyi (1952) method is the most common and widely used method for quantitative determination of reducing sugars. In this method, alkaline copper tartrate and arsenomolybdic acid are used as the major reagents. Reducing sugars reduce copper from cupric (Cu^{2+}) to cuprous state (Cu^{+}) which in turn, reduces molybdic acid into molybdenum, resulting into development of blue colour. OD is measured at 620 nm in a colorimeter.

Preparation of Reagents

(a) Arsenomolybdate colour reagent

Dissolve 25 grams of ammonium molybdate [$(NH_4)_6 Mo_7O_{24} 4H_2O$] in 450 ml of distilled water. Add 21 ml of concentrated H_2SO_4 to it and mix properly. To the above solution, an aqueous solution of sodium arsenate (3g/25ml) is added and mixed. The final solution is placed is an incubator at 37^0 C for 48 hrs.

(b) Alkaline-copper-carbonate-tartrate reagent (Somogyi reagent)

- Dissolve 24 grams of anhydrous sodium carbonate and 12 gram of Rochelle salt (Na-K tartrate) in 250 ml of water. Add 40 ml of 10% copper sulphate solution into the above solution with stirring, followed by addition of 16 gm of sodium bicarbonate (Solution A).
- Dissolve 18 grams of sodium sulphate in 500 ml of hot water and boil to expel air (Solution B).
- On cooling, mix solutions A and B and make final volume upto 1000 ml with distilled water.

(c) Standard glucose solution

Stock: Dissolve 100 mg sucrose in distilled water and make the volume upto 100 ml. Take 10 ml of this stock solution and dilute it to 100 ml distilled water (100 μg/ml). This solution serve as the working standard.

Estimation of Sugars

(I) Sample preparation

Fresh leaf sample (1gm) is collected on clear day and preserved in 95% ethanol in glass vials.

(ii) Extraction of sugars

For sugar analysis, supernatant (alcohol in which the leaf material is plunged) is decanted into a beaker. The extraction is repeated 3 to 4 times by boiling the sample with 50 to 20 ml of 80% (v/v) ethanol in water each time and decanting the supernatant in the same beaker. The combined sugar extract is made upto a final volume of 100 ml with distilled water in a volumetric flask.

(iii) Purification of sugar extract (removal of colloidal particles)

50 ml aliquot of the above sugar extract is evaporated in a water bath taking care not to let the liquid dry out completely. The sample is then treated with one ml saturated solution of lead acetate (to precipitate colloidal substances) and then filtered into a beaker containing 3.0 ml of saturated disodium hydrogen phosphate. Lead is precipitated as lead phosphate. After 2-3 washings the content of beaker is filtered into a 50 ml volumetric flask and made upto a final volume of 50 ml. An aliquot of this solution is used for determining the reducing sugars by Nelson's arsenomolybdate method. Improved copper reagent is used for this purpose.

(iv) Determination of reducing sugars

a). Take 0.2 ml of the aliquot in a test tube and make the volume upto 2 ml with distilled water.

b). Add 1 ml of Somogyi copper reagent and heat in a boiling water bath of 10 minutes.

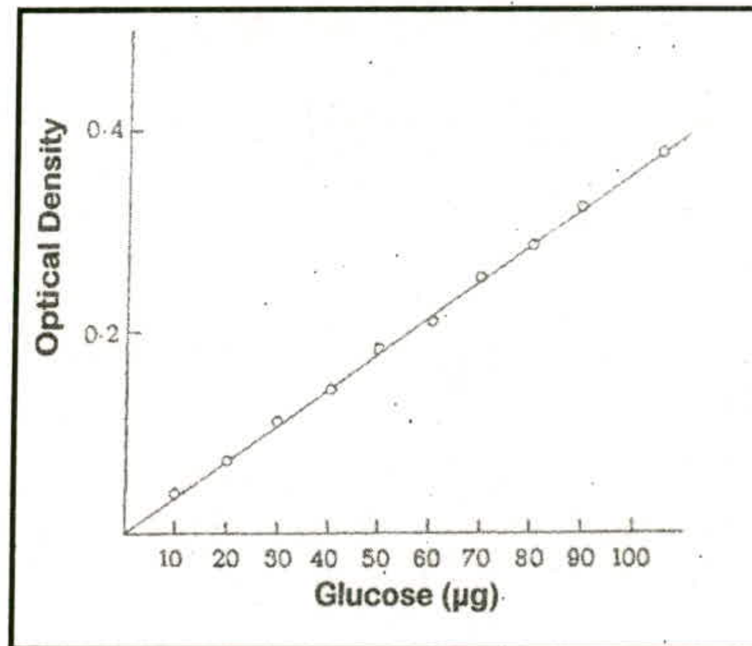
c). After cooling the sample in running tap water, 1 ml of arsenomolybdate reagent is added and final volume made upto 10 ml with distilled water. OD is measured at 530 nm in a spectrophotometer or colorimeter.

d). From the graph drawn (using working standard solutions) calculate the amount of reducing sugars present in the sample.

Calculation

Absorbance corresponds to 0.1 ml of the test solution = x mg of glucose.

100 ml contains $\frac{X}{0.1}$ x100mg of glucose. = % of reducing sugars.



II. Non-Reducing Sugars

Non- reducing sugars are calculated by subtracting the reducing sugars from the total sugar content.

III. Estimation of Total Sugars

5 ml of sugars extract (prepared as above) is hydrolysed by boiling with 2.5 ml of 0.5 N-HCl for 30 minutes in a water bath and later neutralized to slightly acidic side with 0.5 N-NaOH. The final volume is made upto 10 ml with distilled water and the solution is used for determining total sugars. An aliquot of above solution is analysed for total sugars as described in step c. OD is measured at 530 nm.

PRACTICAL NO. 15

Object: Estimation of Starch by Anthrone Reagent

Principle

The sample is treated with 80% alcohol to remove sugars and then starch is extracted with perchloric acid. In hot acidic medium starch is hydrolyzed to glucose and dehydrate to hydrozymethyl furfural. This compound forms a green coloured product with anthrone.

Material required

Anthrone: Dissolve 200 mg anthrone in 100 ml of ice-cold 95% sulphuric acid, 80% ethanol, 52% perchloric acid and Standard glucose: Stock - 100 mg in 100 ml water. Working standard 10 ml of stock diluted to 100 ml with water.

Procedure

1. Homogenize 0.1 - 0.5 g of the sample in hot 80% ethanol to remove sugars. Centrifuge and retain the residue. Wash the residue repeatedly with hot 80% ethanol till the washings do not give colour with anthrone reagent. Dry the residue well over a water bath.
2. To the residue add 5.0 ml of water and 6.5 ml of 52% perchloric acid.
3. Extract at 0^0C for 20 min. centrifuge and save the supernatant.
4. Repeat the extraction using fresh perchloric acid. Centrifuge and pool the supernatants and make up to 100 ml.
5. Pipette out 0.1 or 0.2 ml of the supernatant and make up the volume to 1 ml will water.
6. Prepare the standards by taking 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard and make up the volume to 1 ml in each tube with water.
7. Add 4 ml of anthrone reagent to each tube.
8. Heat for eight minutes in a boiling water bath.
9. Cool rapidly and read the intensity of green to dark green colour at 630 nm.

Calculation

Find out the glucose content in the sample using the standard graph. Multiply the value by a factor 0.9 to arrive at the starch content.

PRACTICAL NO. 16

Object: Estimation of Total Free Amino Acid

Sample preparation

Five grams of plant material is weighted and ground in a mortar-pestle with 10 ml of ethanol. The crushed material is centrifuge and the supernatant is carefully transferred. The residue is again ground with10 ml of ethanol and treated as above. The extraction is done three times to obtain total free amino acids. The final volume is made up to 50 ml with ethanol and used for estimation of the total free amino acids.

Preparation reagents

(i) *Citrate buffer:* 0.5 M (pH 5.6), (ii) *Ninhydrin:* 1% solution in 0.5 M citrate buffer (pH 5.6), (iii) *Glycerol:* 55% solution of glycerol is prepared in distilled water.

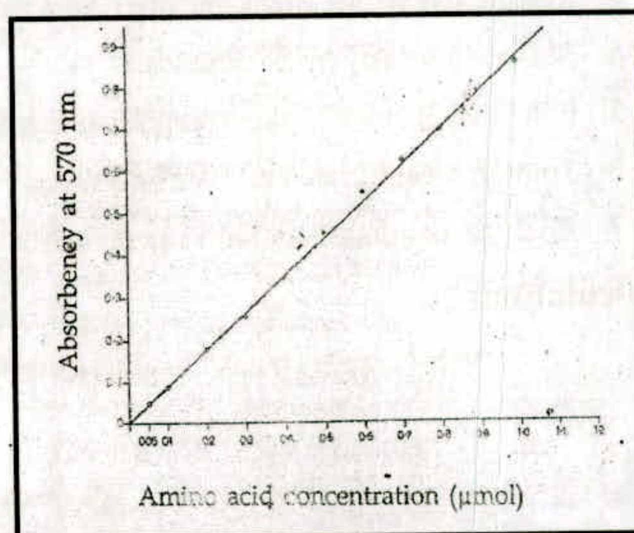
Procedure

Following reagents are taken in test tube in duplicate (I) 0.2 ml ninhydrin solution (ii) 0.2 ml Citrate buffer (iii) 1.2 ml Glycerol (iv) 0.1 ml of sample solution (in control, sample solution is not added)

The volume of the mixture is made upto 5 ml by adding distilled water. Test tube are then heated in a boiling water bath for 30 minutes, cooled in running tap water and gently shaken. The absorbance is read using a spectrophotometer at 570 nm. A blank/control is also taken simultaneously for background correction. The quantity of total free amino acid is computed by referring to standard curve prepared from glycine.

Preparation of standard curve for total free amino acid

Solution of different concentrations are prepare from a stock solution of glycine 10 µM per 0.1 ml. Solutions are put in test tubes containing the reagents mentioned above and processed as describe earlier. A blank/control (without glycine) is also run alongwith other solutions for background correction. Standard curve is plotted on the readings obtain for different concentrations of glycine at 570 nm.



PRACTICAL NO. 17

Object: Estimation of Extractable Nitrates

Reagents and apparatus

- *Extracting solution:* Add about 600 ml of distilled water to a 1 liter volumetric flask. Add 20.0 ml of glacial acid in the same flask. Bring to final volume with distilled water. Mix and transfer to a plastic storage bottle.
- *Nitrate standard stock solution:* Dry the potassium nitrate in the oven for 2 hours. Weigh 0.722 g of potassium nitrate and transfer to a 1000 ml volumetric flask. Bring to volume with the extracting solution. Label "100 ppm NO_3-N in 2% acetic acid extracting solution.
- *Working solution:* Set of four working NO_3. Standards: 20.0, 10.0, 5.0, 1.0 mg N/L by volume: To four 100 ml of the nitrate standard stock solution. Dilute each to the mark with the working solution and invert to mix.
- 125 ml shaking bottles.
- 10 ml testing tubes
- Whatman # 2 filter paper without nitrates.

Procedure

1. Weigh out 0.100 g of dry ground plant material into each 125 ml shaking bottle.
2. Add 25 ml of extracting solution into each flask, insuring that all plant material has been wetted.
3. Include at least one Blank and one Check sample per run.
4. Shake vigorously for 15 minutes.
5. Filter into 30 ml of beaker using Whatman #2 filter paper without nitrates.
6. Transfer clear filtrate into 10 ml test tubes.
7. Analyze for nitrate on a Flow Injection Analyzer.

Calculations

NO_3-N ppm in plant = reading (ppm) x 25/0.100

PRACTICAL NO. 18

Object: Protein Estimation by Lowry's Method

The method combine the reactions of copper ions with the peptide bonds under alkaline conditions (the Biuret test) with the oxidation of aromatic protein residues. The Lowry method is best used with protein concentrations of 0.01 - 1.0 mg/ml and is based on the reaction Cu+, produced by the oxidation of peptide bonds, with Folin-Ciocalteu reagent (a mixture of phosphotungstic acid and phosphomolybdic acid in the Folin-Ciocalteu reaction). The reaction involved reduction of the Folin reagent and oxidation of aromatic residues (mainly tryptophan, also tyrosine). Experiments have shown that cysteine is also reactive to the regent. Therefore, cysteine residues in protein probably also contribute to the absorbance seen in the Lowry Assay. The concentration of the reduced Folin reagent is measured by absorbance at 750 nm. As a result, the total concentration of protein in the sample can be deduced from the concentration of Trp and Tyr residues that reduce the Folin reagent.

Material required

Alkaline sodium carbonate solution: it is prepared by dissolving 2.0 g of anhydrous sodium carbonate in 100 ml of 0.1 N NaOH solution.

Alkaline cooper reagent: (a) It is prepared by dissolving 0.5 g of pentahydrate copper sulphate in 1.0% solution of sodium potassium tartarate. Mixing of the two solution is done fresh before use (b) 1.0 ml of copper reagent is mixed with 50.0 ml of alkaline sodium carbonate solution.

Follin - Ciocalteau reagent: This is obtained commercially and it contain sodium tungstate, sodium molybdate in phosphoric and hydrochloric acid. This is diluted to 1:2 with distilled water.

Protein solution (stock solution): 50.0 mg of bovine serum albumin (Fraction V) is dissolved in distilled and volume is made to 50.0 ml.

Working standard: 10.0 ml of stock solution of protein in diluted to 50.0 ml with water and this solution contain 200 mg/ml.

Procedure

1. Extraction of soluble proteins from green leaves or germinating seeds is usually carried out with 0.2M phosphate buffer pH 7.5, centrifuged and supernatant is used as protein source.
2. To 0.1 ml of protein extract, 1.0 alkaline copper reagent is mixed and kept in room temperature for 10 minutes and thereafter 0.1 of Folin-Ciocalteau reagent is added and colour is allowed to development for 30 minutes.
3. After 30 minutes, the final volume is made to 5.0 ml with water and optical density is recorded

at 750 nm against reagent blank where 0.1 ml of protein extract is replaced with water.

4. A gradient of soluble protein (20-200 mg/ml) is taken from the working stock solution for protein and colour is developed in accordance to the method described above. Standard graph is obtained by plotting the optical densities against respective protein concentrations.
5. Soluble protein content of the sample is calculated after referring the standard curve and is expressed as mg of protein/g fresh weight.

NOTE

- Fore complete enzyme extraction, sometime the chemicals like ethylenediamine tetraacetic acid (EDTA), magnesium salts and mercaptoethanol are included. This method of protein estimation should not be followed if the extractant contains K+, Mg++ Tris, EDTA and thiol (mercaptoethanol) compounds as they interfere with this procedure. When these chemicals are present in the extract, protein is precipitated by the addition of 10% TCA and the precipitated protein is centrifuged out and redissolved in 2.0 N NaOH for protein estimation.
- Follin-Ciocalteau reagent can be prepared in the laboratory and for this refluex gently for 10 hours a mixture having 100g sodium tungstate ($Na_2MoO_4.2H_2O$), 25 g of sodium molybdate ($NaMoO_2H_2O$), 700 ml water, 50.0 ml of 85% phosphoric acid and 100.0 ml of concentrated hydrochloric acid. Lithium sulphide 150.0 g water 50.0 ml and few drops of bromine water is added after reflux. Boil the mixture for 15 minutes without condenser to remove excess of bromine. Cool, dilute to 1000 ml and filter. The regent should have no green tint.
- If protein estimation is desired in a sample with phenolic or pigment content, extract should be prepared with a reducing agent preferably cystein and NaCl. Precipitate the protein with 10% TCA, seprate the protein and redissolve in 2.0 N NaOH.

PRACTICAL NO. 19

Object: To Study the Photoperiodic Response of Plants

The role of light has been studied in photosynthesis, growth and development where the intensity and quality of light play an important role. Similarly the length of the daylight period has been found to have a marked influence on the behavior of plants particularly flowering. The observation of Garner and Allard (1920) that a mutant variety of tobacco, Maryland mammoth flowers when the relative length of photoperiodism. They use the term photoperiodism to designate the response of an organism to the relative length of day and night and photoperiod to designate the favourable length of day for each plant. Thus the phenomenon in which the influence of day length on plants is studied is called photoperiodism. "It may also be defined as the response of plants to the relative length of day and night." On the basis of the length of photoperiod requirement, they have classified plants into: (i) Short-day plants (ii) Long-day plants (iii) Day-neutral plants **(Fig 19.1)**.

(i) Short-day Plants

For flowering of short-day plants the day length must not exceed a certain critical value, the day length required is less than a certain critical length. Short-day plants may be more correctly called long night plants as a certain minimum of uninterrupted dark period in 24 hours is necessary for their flowering.

If the dark period is less than a critical length, flowering will not occur. Short-day plants will not flower even if a flash of light or weak light is provided during the continuous dark period. However, the light interruption is not very effective if it is nearing the beginning or the end of the dark period. These plants are also not capable of flowering if short dark and short light are provided alternately. Hillman (1959) showed that short-day plant are capable of flowering even if kept continuously in the dark but provided with sucrose. This shows that the short-day plants require light period only for carrying on photosynthesis. In short-day plants flowering can be induced even during long days by increasing the dark period by putting them in the dark for some time before sunset or after sunrise. In these plants cutting short of the light period up to 12 hours or less induces flowering in them. Some examples of short-day plants are soybean, poinsettia, potato, sugarcane, cosmos, chrysanthemum, aster, dahlia, tobacco (Maryland mammoth), cocklebur (Xanthium strumarium), rice, hemp, datura, onion, upland cotton (Gossypium hirsutum), strawberry etc.

(ii) Long-day Plants

Examples of long-day plants are spinach, lettuce, radish, alfalfa, sugar beet, opium poppy, larkspur, oats, henbane, wheat etc. Long-day plants require photoperiod of more than a critical length which may vary from 14 to 18 hours. The best flowering of long day plants usually occurs in continuous light. For flowering they require either no dark period or a very short dark period. A flash

of light given to long-day plants during long dark periods can induce flowering in them even during short day periods. Here, darkness has an inhibitory effect on flowering. The long day plant can flower even in short-day periods if these short-day periods are accompanied with still shorter dark periods. A long-day plant requiring 16 hours of light period in 24 hours can be made to flower if it is provided with a cycle of eight hours of light period and four hourse of dark period. The flowering in long-day plants is inhibited not because of the short light periods but because of the too long dark periods and because of this, long-day plants can also be called short-night plants.

(iii) Day-neutral Plants

Tomato, cucumbers, cotton, pea, sunflower, maize, dandelion etc. are examples of day-neutral plants. Their flowering is not affected by the length of the day. They can flower even if the light period provided is from few hours to continuous illumination.

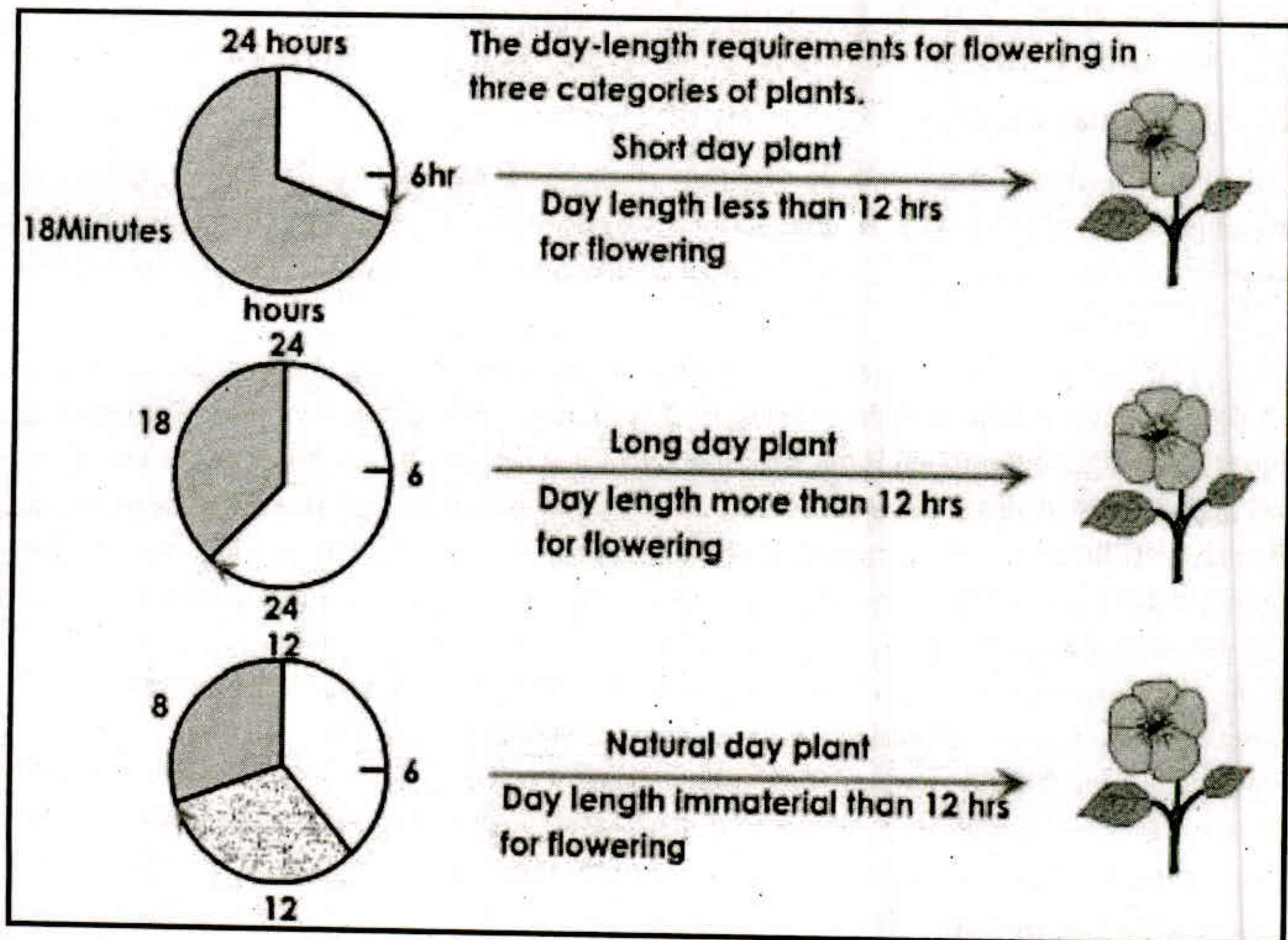


Fig 19.1 (i) Short-day plants (ii) Long-day plants (iii) Day-neutral plants

PRACTICAL NO. 20

Object: Bioassay for Different Growth Hormones

Growth regulators define as organic compounds synthesized in one part of a plant transported to another where at very low concentration they cause physilogical response. There are five major groups of growth regulators namely, the Auxin, Gibberellins, Cytokinins, Abscissic acid (ABA) and Ethylene.

(A) Wheat coleoptiles straight growth bioassay for Auxin

Principle

The physiological basis for straight growth test is the stimulation of cell elongation by auxins. The increase in length of coleoptiles will be propotional to the auxin levels in the medium. The increase in length is then compared with that of the coleoptiles floated over buffer.

Material Required

Solutions and buffers, wheat seed, sandy trays, a sharp razor blade, a scale to measure the length of coleoptiles.

Solutions and buffers used for the experiment

Acetate buffer (pH 5.6): (Sodium acetate:acetic acid)

- Solution A: Prepare 0.2 M sodium acetate $3H_2O$ by dissolving 2.772 g and make up the volume to 100 ml.
- Solution B: Dilute 1.2 ml of of glacial acetic acid to 100 ml with distilled water to obtain 0.2 M acetic acid.To 91 ml of solution A add 9 ml of solution B to prepare 100 ml of acetate buffer of pH 5.6. Use this buffer to prepare the GA solutions as well as for the exptraction of the enzyme from the soaked seed.

Procedure

1. Germinate wheat seeds on moist filter paper and transfer them on to sand or on vermiculite. Allow the seedlings to grow a temperature of 24-26^{0}C under high humid conditions for a period of 73 to 98 hrs in darkness.
2. When the colioptiles have attained a length of 20 to 30 mm, detop the coleoptiles in darkness and allow the auxins to diffuse down in 4 to 5 hrs.
3. From the top portion of the coleoptiles (just below the emerged primary leaf) cut 5 mm long segments using a sharp razor blade.
4. Place five coleopltiles sements in each of the different concentrations of auxin ranging from 10-

7 to 10-4 M (the different auxins concentrations can be serially diluted from a 10-3 M stock solution). Similarly place coleoptiles segments in the test solution.

5. Float 5 coleoptile segments on the buffer used for preparing the auxins solutions to serve as control.
6. Measure the length of the segments after 24 hrs and compare with that of the coleoptiles floated over the buffer.
7. Calculate the percentage incerase in length over control and plot the values against the respective auxins concentrations (you may also plot the absolute values of the length of the coleoptiles against the auxins concentrations).
8. Similarly, incubate coleoptiles in test solution and measure the increase in length.
9. Compare the increase with the standard curve and estimate the levels of IAA in the test solution

(B) Dwarf rice seedling elongation bioassay for Gibberellin

Principle

Dwarf rice, Tan-ginbozu, lacks the capacity to synthesize the specific GA (GA_1) necessary for stem elongation. The stem of these seedlings respond to the external supply of GA and elongate as their normal counterparts. The extent of elongation of dwarf seedlings will therefore the proportional to the concentration of GA supplied.

Material Required

Tan-ginbozu (Dwarf rice) seed, filter paper, buffers and solutions (See below for procedure), 25 ml beaker, humid chambers, scale to measure the length, Acetate buffer (pH 5.6): (Sodium acetate:acetic acid).

- Solution A: Prepare 0.2 M sodium acetate.$3H_2O$ by dissolving 2.772 g and make up the volume to 100 ml.
- Solution B: Dilute 1.2 ml of of glacial acetic acid to 100 ml with distilled water to obtain 0.2 M acetic acid. To 91 ml of solution A add 9 ml of solution B to prepare 100 ml of acetate buffer of pH 5.6. Use this buffer to prepare the GA solutions as well as for the exptraction of the enzyme from the soaked seed.
- GA stock solution (10-3 M): Prepare a 10-3 M GA stock solution of GA using of acetate buffer. Weigh 34.2 mg of GA_3 and dissolve it in a little amount of alcohal and make up the volume to 100 ml with buffer.
- Agar gels: Weigh 1 g of Agar and mix it in 100 ml of distilled water. Boil the mixture until the agar completely dissolves in water. When the agar solution is cooled it gets solidified to form a gel. Appropriate aliquate of GA stock solution was added to the agar before

solidification to obtain agar gels with different concentrations of GA. Similarly, the test solution was added to the agar solution after appropiate dilutions.

Procedure

1. Germinate Tan-ginbozu seeds on moist filter paper paper in darkness.
2. Pour the agar solutions with different concentrations of GA ranging from 10-4 to 10-10 M into 20 ml beakers and allow solidifying.
3. Place a few germinated seeds on each of these agar beakers. Keep the beakers in a humid chamber at room and allow the seedlings to grow for a week to 10 days.
4. Measure the length of each of the seedling in all the beakers and estimate the average stem length in cm.
5. Maintain a set of seedling place on agar gels without any GA to serve as control.
6. Plot the length of the seedlings as afunction of their respective GA concentrations.

(C) Retardation of leaf senescence bioassay for Cytokinin

Cytokinins are substitute amino acid purines that promote cell division in callus tissuse in presence of optimum concentration of auxins. Apart from this function, cytokinins have several others formative effect. All biologically occurring cytokinins are trans forms and the most commonly occurring form of cytokinin is trans Zeatin riboside (t-ZR).

Procedure

The equal size of pieces of barley, oat, tobacco or any other leaf material *i.e.* excised leaf sections or discs are kept on test solution (with different concentration of kinetin) moistened filter papers for 48 hrs and then chlorophyll retention or loss prevented by cytokinin is recorded.

Table 20.1: Based on formative effect of cytokinins various bioassay are developed.

Bioassay	Min. Detectable level	Range
Tobacco callus bioassay	5×10^{-8} M	5×10^{-9} M – 10^{-7}
Carrot explants bioassay	5×10^{-9} M	5×10^{-9} M – 10^{-7}
Radish cotyledon bioassay	5×10^{-8} M	5×10^{-8} M – 10^{-6}
Cucumber cotyledon bioassay	5×10^{-7} M	5×10^{-8} M – 10^{-6}
Barley root growth bioassay	5×10^{-8} M	5×10^{-8} M – 10^{-5}

Precautions

The cucumber bioassay is generally not specific to cytokinins alone. High concetrations of GA can also elicit a similar response of cotylendons expansion. However, a concentration of 25 to 50 mM of K+ is necessary for the cotyledon expansion. A very high level of syngergistic effect is

also seen between K^+ and cytokinins in cotyledon expansion. Therefore, it is necessary to eliminate K^+ from the assay medium or the control set also should have the same concentration of K+ as that of the test solution.

a. Keep a control where the cotyledons are kept in the buffer without cytokinins.

b. Carefully blot the cotyledons to dryness and record their fresh weight. Compute the per cent increase in fresh weight over the control value.

c. Tabulate and plot the results and write comments.

(D) Bioassay of Abscissic acid (ABA)

ABA is a general plant growth inhibitor. The method of ABA estimation in leaves by using High Performance Liquid Chromatography (HPLC).

Sample collection

Fully expanded leaves should be collected in butter paper bags and kept in ice box. The samples are preserved in liquid nitrogen for further analysis.

Extraction

Take leaf sample (2-5 g) in a flask and pour so much volume of containing 1% acetic acid that the sample dips into it completely

Incubate it overnight at 4°C

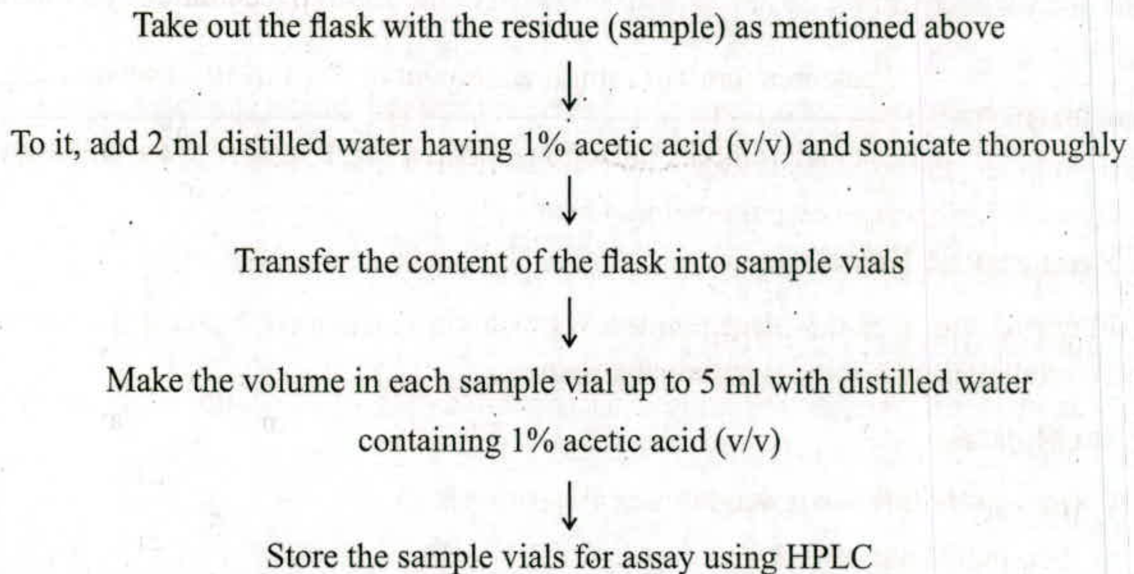
Filter the extract through a filter paper (Whatman No.4)

The extraction should be repeated twice or thrice (*i.e.* repeat the above steps 2-3 times, each time use the residue on the filter paper for extraction

Evaporated acetone from the total volume of extract using a rotary evaporator at 40-50°C till some residue is left on the bottom

Preserve the residue in a cool and dark place

Sample preparation



The analysis of ABA using HPLC are maintained as follows:

Column	:	Reverse phase C18 column having particle size equal to 5µm
Detector	:	UV detector
Wavelength	:	265nm
Flow rate	:	1.5 ml/min
Calibration curve	:	Using 10 ppm solution of ABA in 95% ethyle alcohal

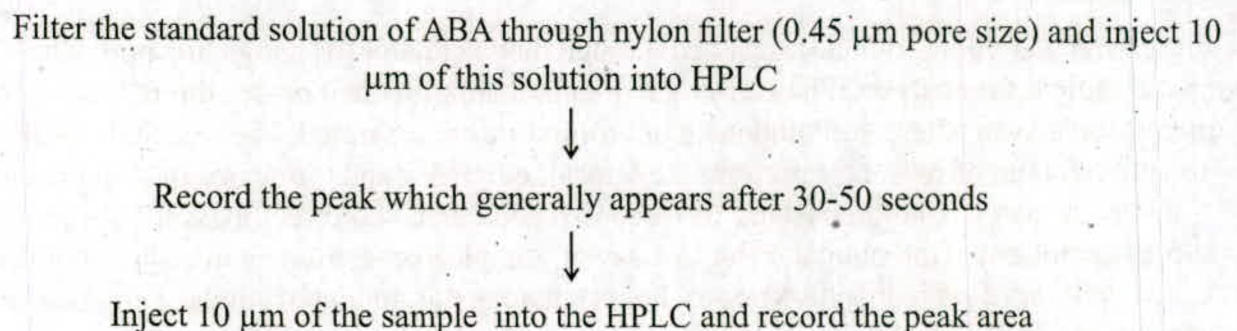
Solvents

A : 1% acetic acid in distilled water (HPCL grade) v/v

B : 1% acetic acid in methyl alcohal (HPCL grade) v/v

Both the solvents are filtered through millipore nylon filter (13µm) with the help of vacuum pump solvent filter kit.

Assay



The ABA content in the sample may be calculated using the equation given below:

$$\text{ABA content (ng)} = \frac{\text{Peak area (cm}^2\text{) of sample x Amount of ABA in 10 } \mu\text{l solution (ng)}}{\text{Peak area (cm}^2\text{) of standard solution x Fresh weight of the leaf sample (g)}}$$

(E) Bioassay of Ethylene

Ethylene is one of the plant hormones which are extensively quantified using physico-chemical techniques such as the Gas chromatography.

Sample collection

1. Put mango fruits (known weight) in a cylinder or jar.
2. Seal the mouth with a gasket.
3. Incubate for at least 2-4 hrs at room temperature.
4. Withdraw the gas sample (ethylene) using a hypodermic syringe for assay. A known volume of gas should be assayed.

Ideal operating conditions for gas chromatograph

- Carrier gas : Hydrogen/Nitrogen mixture
- Flow rate : 20-30 ml/min.
- Gas for detector : Hydrogen and air
- Column : Porapak-Q 80/100 mesh packed
- Oven/coumn temperature : 60^0C
- Injector temperature : 110^0C
- Detector temperature : 85^0C
- Detector (to be used) : FID (Flame Ionization Detector)
- Retention time for ethylene : 1.3 min.

Procedure

The carrier gas from a cylinder is passed through flow regulator to an injection port, where it picks up the sample for analysis. The carrier gas + sample mixture then passes through the coloumn in thermostatic oven where the component of the mixture are separated. The area of the peak depends upon the amount of substance present, the detector efficiency and the degree of amplification used. If the latter factor is hold constant, the recorded peak area is a direct measure of the amount of substance present in the sample. Prior to assay of sample, the instrument must be calibrated with a known volume of standard ethylene gas. For calculating the amount of ethylene produced per gram of sample per unit time.