LABORATORY MANUAL

for

5-Year Integrated *(B.Sc. - M. Sc.)* Botany

PLANT PHYSIOLOGY

DEPARTMENT OF BOTANY

UNIVERSITY OF KASHMIR, NORTH CAMPUS,

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LIST OF EXPERIMENTS:

1. Determination of osmatic potential of plant cell sap by plasmolytic method.
2. To study the effect of environmental factors (light and wind) on transpiration of excised twigs.
3. Calculation of stomatal index and frequency of a mesophyte and a xerophyte.
4. To demonstrate the ascent of sap in young plant seedlings using eosin solution.
5. Demonstration of Hill reaction.
6. Separation of photosynthetic pigments by paper chromatography.
7. Separation of photosynthetic pigments by thin layer chromatography (TLC).

The following basic steps should be observed at all times in the laboratory:

1. Upon entering the laboratory, place your belongings in specified locations, never on bench tops.
2. At the beginning and termination of each laboratory session, wipe bench tops with a disinfectant solution.
3. Do handle slides and specimens with care.
4. Wash your hands with liquid detergent and disinfectant upon entering and prior to leaving the laboratory.
5. A laboratory coat is necessary while working in the laboratory, to protect clothing from contamination or accidental discoloration by staining solutions.
6. Wear disposable gloves during laboratory operations.
7. Tie back long hair to minimize its exposure to open flames, wear closed shoes at all times in the laboratory setting, never apply cosmetics or insert contact lenses in the laboratory.
8. Do not eat, or drink in the laboratory.
9. Don't directly touch any chemical with hand
10. Report accidental cuts or burns to the instructor immediately.
11. Speak quietly and avoid unnecessary movement around the laboratory to prevent distractions that may cause accidents.

EXPERIMENT 1.

Determination of osmatic potential of plant cell sap by plasmolytic method.

A metabolically active plant cell contains about 75-95% water by weight. Water is important for proper growth and development of plants. Any significant reduction in its availability can affect the metabolic activity and which in turn affects growth and yield. The uptake of water by the plant roots from the surrounding environment (soil solution) is governed by water potential. Water potential represent the free energy per mole of water and is measured in bars or atmospheric pressure. It is symbolized by the Greek word (Psi Ψ and expressed as force/unit area (1 bar = 10^{-1} Pa = 10^{-6} dynes cm^2 = 0.99 atmosphere or 10 J kg^{-1}). The water potential of an unbounded solution is exactly equal to the osmotic potential of that solution, i.e. Water potential (Ψ) = Osmotic potential (Ψ_0).Water potential may be reduced by:

I. Decreasing the temperature
II. Adding soluble solute
III. Decreasing pressure or
IV. Providing additional absorptive surface

The water potential of pure water is zero. Therefore, any of the above change could result in a negative water potential and affect water uptake by plants.

PRINCIPLE

The water potential of a cell and its ability to take up water is a function of osmotic potential affected by the solutes in the vascular cell sap. If the osmotic potential of a flaccid cell is lower than that of external environment the cell will take up water until the pressure is at equilibrium with that or the external solution or until the rigid cell wall creates a resistance too high to allow additional expansion. This internal pressure is the turgor pressure and represents the real pressure within a cell whereas osmotic pressure represents only the potential for pressure that could be created by the cell is equal to the osmotic potential + turgor pressure.

The movement of water into a cell is a function of the difference between the water potential of outside and inside of the cell. The direction of movement will always be towards the lower water potential and the difference will indicate the force with which the water will enter into the cell.

If a cell is placed in hypersonic solution it will lose water and become plasmolysed. If a cell is placed in hypotonic solution it will take up water and will become turgid. In an isotonic

solution, the cell will be at equilibrium and the cell will be at the state of incipient plasmolysis. At this point the water potential is always equal to the osmotic pressure potential since there is no turgor pressure.

Methods of measuring osmotic potential

There are different methods that have been developed to measure the osmotic potential of cells or solutions. The techniques basically are either physical or biological.

(a) Physical method

This method is based on the ability of the cellular solutes to change the physical properties or the pure water such as change in freezing point depression or change in vapour pressure. 1 M sucrose solution has an osmotic potential of approximately -22.4 bars and will depress the freezing point of water by -l.86°C. Since this change in the freezing point is proportional to the change in the osmotic concentration and pressure potential, the temperature depression of the freezing point will allow the calculation of the osmotic potential. A similar method is used based upon the change in the vapour pressure of water that is effected by the addition of solute from the cell.

Drawback: To obtain the cell solutes the cell arc ground up and thus it is destructive method that adds the adsorptive surfaces of the cytoplasm to those of the cell sap.

(b) Biological method

It involves the experimental estimations of the concentration of an external solution which will affect the state of incipient plasmolysis at which there is no turgor pressure. Therefore, the osmotic potential of a cell is equal to that of the external solution. Since, the osmotic potential of the external solution is proportioned to its osmotic concentration (1 M sucrose = -22.4 bars), the osmotic potential can readily be calculated once the osmotic concentration has been determined.

MATERIALS REQUIRED

Sucrose, microscope, leaf material *(Rhoeo discolor),* petri-plates, slides and cover slips.

PROCEDURE

1. Take 1 or 2 ml of the following sucrose stock solution and set up a concentration series in the petri plates (0.14, 0.16, 0.18, 0.20, 0.22, 0.24, 0.26, 0.28 and 0.30 M).
2. Peel out the purple lower leaf surface of the leaf having unicellular strips (see that the strips should contain sufficient number of intact cells and the red anthocyanin pigment is present).
3. Put these strips of cells in the solution of different concentrations.

4. After 30 minutes, put the strip on a microscope slide along with some sugar solution of the same concentration.
5. Use the cover slip so that it may not dry up. Examine about 25 of the intact cells (selected randomly) and record the number of plasmolysed and non-plasmolysed cells for each solution.
6. Count the cells showing any separation of the plasma lemma from the cell wall as plasmolysed.
7. Record the observation in a tabular from as given below:

Sucrose solution	Plasmolysed cells	Non-plasmolysed cells	% of plasmolysed cells
0.14 M			
0.16 M			
0.18 M			
0.20 M			
0.22 M			
0.24 M			
0.26 M			
0.28 M			
0.30 M			

Plot the above data on a graph paper. The solution concentration at which 50% of the cells are plasmolysed is considered to be the one that causes incipient plasmolysis.

If someone wants to be more specific, one can further dilute the solution. Suppose we get 0.22 M solution concentration. Where we get incipient plasmolysis, we can further dilute the solution as given below:

Sucrose solution	Plasmolysed cells	Non-plasmolysed cells	% of plasmolysed cells
0.200 M			
0.205 M			
0.210 M			

0.215 M			
0.220 M			
0.225 M			
0.230 M			

Now calculate the osmotic potential (Ψ) of cells using the following formula:

Ψ_0 = -22.4/273 x MxT

M = Molar concentration (moles/litre) or the external solution that causes incipient plasmolysis

T = Absolute temperature (up to 273 = 0°C) = (273 + room temperature in °C)

Also compare it by using the Van't Hoff law:

Ψ_0 = - miRT

Where; m =Molality

i = Ionization constant (1.0 for sucrose)

R = Raul's gas constant (0.083 liter bars/mole)

T = Temperature (273 + room temp. in °C)

PRECAUTIONS

1. Use exact solution gradient concentration.
2. Do not allow the cells to dry during cell counting.
3. Use clean slides and cover slips.

EXPERIMENT 2

To study the effect of environmental factors (light and wind) on transpiration of an excised twig.

Principle:

Transpiration is the process by means of which excess water present in plant tissue eliminates in the form of water vapour through the aerial parts of plants like twig or leaf etc. The stomata constitute the main organ for land plants. The rate of transpiration depends on the different factor such as light, wind, humidity etc.

Requirements:

Plant material - A twig of *Ficus* leaf.

Glass Ware - Conical flask -2 pcs. 250 ml.

Glass rod - 2pcs.

Chemical – Oil.

Instruments - Digital weight machine Graph paper, Pencil, Thread, blotting paper etc.

Procedure

Two conical flasks were filled with distilled water upto their neck. Two petiolate leaves were cut under water from the twig .Then immediately inserted into flask, their petioles remain under water. Now oil was poured to cover the upper surface of water to check the evaporation. The whole sets were weighted and one set was allowed to stay in open air with the sufficient light and another set was kept in dark condition for two hours. After two hours two sets were reweighted. The difference between two weights is the amount of water loss. The total transpiration areas are measured by a graph paper method.

Result

The rate of the transpiration under air with the sufficient light = mg/sq./hr.

Dark condition = mg/sq./hr.

Precaution:

- The leaf should be fresh and clean.
- Oil should cover the whole upper surface of water.
- All weight should be taken carefully.
- Only petiole should be under water.

- When blowing of wind started having chance of leaf fall from the set, it should be checked.

Discussion

The rate of the transpiration is directly depends on the velocity of the wind and light. The rate of the transpiration is increased due to the effect of wind velocity and light. As the wind flows, it accelerates the transpiration from the surface of the leaf compare to dark condition of set.

Condition of set	**Initial wt. in grams**	**Final wt. in grams**	**Difference of wt. in grams**	**Total area in sq.**	**Time**	**Rate of transpiration**
In wind velocity & light						
Dark condition						

EXPERIMENT 3

Calculation of stomatal index and frequency in plants

PRINCIPLE:

Surface anatomical features could be studied under light microscope either by directly observing the epidermal peel or initially making the replica of the leaf surface and observing the same under the microscope. Replica method was developed by Meidner and Mensfield (1968) and Wolf el al. (1979).

MATERIALS REQUIRED:

Leaves, replica fluid, brush, forceps, glass slides, cover slips, needles, microscope, ocular meter. Stage micrometer etc.

METHODOLOGY:

A. Preparation of replica fluid

Commercially available, clear nail polish, red/blue correction fluid as used for making changes in micrograph stencil or quick fix (adhesive) can be used for replica preparation. Celludin (8%), or cellulose acetate in acetone can also be used for taking imprint. Now a days imprint on silica rubber or poly-methyl-meta-rylate (PMMA) combined with replicas by cyanacrylate adhesive are being used.

B. Calibration of ocular micrometer

1. Place the stage micrometer (SM) on the stage of the microscope and focus it at a given magnification e.g. 40x. SM is a calibrated scale having 100 division of 1 mm and therefore 1 division is equal to 0.01 mm or 10 μm or micro-meter.
2. Place the ocular micrometer (OM) in one of the eye pieces of microscope. Align the scale of OM with that of SM while observing through the eye pieces.
3. Count the divisions of OM coinciding with divisions of SM. (For example: 4.0 divisions of OM = 45 divisions of SM i.e. 450 μm 1 division of OM = 11.25 μm).
4. Following the above three steps calibrate the ocular scale for other magnifications also i.e. 60x, 100x etc.
5. Once the ocular scale is calibrated for different or required magnifications, SM can be removed and slide having sample material may be placed for observations and measurements.

C. Determination of stomatal density

Collect the suitable leaf material, wash them gently with running water to remove the dust and debris and allow them to dry. If peeling of epidermal layer is possible then it can be used directly otherwise, apply the suitable replica fluid like quick fix (an adhesive) and spread it on the leaf surface and allow it to dry. Gently peel off the replica with the help of forceps or fingers and place it on the slide in a manner that imprint surface should be on upper side. Put one or two drops of water for proper spreading of replica and cover it with cover slip. Similar preparations can be made for different leaves for their upper and lower surfaces. Slides are now ready for calculating the stomatal density which means number of stomata / mm^2.

At a given magnification count the total number of stomata as visible in circular view field or microscope. Calculate the diameter of view field by ocular scale. Now the area of circle under microscopic view field could be calculated by following formula:

$$\pi r^2$$

Where, r is the radius of the circle (view field) i.e. 1/2 of the diameter of circle. Now calculate the number of stomata for mm^2 area of upper and lower surface of leaves.

D. Determination of stomatal index

Slides as prepared for stomatal density are used for determination of stomatal index by using following formula at a given magnification:

Stomatal index (%) = S x 100 / S+E

Where, S and E are the number of stomata and epidermal cells respectively in microscopic view field. Like stomatal density, stomatal index (%) can be calculated for both the surfaces of leaves.

EXPERIMENT 4

Demonstrate ascent of sap using eosin solution

Theory:

The ascent of sap in plants refers to the process through which water and dissolved minerals move from the roots up through the plant to the leaves. This movement occurs primarily through the xylem tissues and is driven by processes like transpiration, capillary action, and cohesion and adhesion properties of water.

Requirements:

Fresh leafy shoot of balsam or petunia, blade, eosin dye, distilled water, scissor, stand, beaker, cotton, weighing balance, cotton etc.

Procedure:

1. A leafy shoot of petunia or balsam was cut under water obliquely.
2. Dipped the cut end in 2% eosin solution contained in a beaker.
3. Adjusted the shoot erect using a stand, cotton and left the set up undisturbed for an hour.
4. Observed the color of veins on leaves, stem and flower after sometime.
5. Cut T.S of stem and V.S of leaf for observing the path of ascent of sap.

Precautions:

1. The leafy shoot should be cut under water obliquely.
2. Eosin solution should be prepared carefully.
3. The transverse section should be thin enough.

EXPERIMENT 5

Demonstration of Hill reaction

Principle:

One of the first steps of photosynthesis is the photolysis of water to donate two electrons to the reaction center, P680. This reaction is known as the Hill reaction. The electrons donated to P680 move through an electron transport chain to the reaction center P700, and eventually, to reduce NADP to NADPH. The complete reaction can be summarized as follows:

$$H_2O + NADP \xrightarrow[\text{Chloroplast}]{\text{Light}} NADPH + 1/2\,O_2 + H^+$$

Other electron acceptors can be substituted for NADP, which allow the Hill reaction to be measured, and some of its components studied. The most commonly used electron acceptor is the dye 2,6 dichlorophenol indophenol (DCIP), which can accept electrons instead of P700. When DCIP is added to a chloroplast or thylakoid suspension, the following reaction occurs:

$$H_2O + DCIP \xrightarrow[\text{Photosystem II}]{\text{Light}} DCIP\ (\text{reduced}) + 1/2\,O_2$$

DCIP is a blue color in its oxidized form. When reduced, it is colorless. This property of DCIP allows the measurement of the rate of the Hill reaction.

Reagents Required:

I. Potassium phosphate buffer: 125 mM pH 7.5 with 0.3 M sucrose.

II. DCIP: 30 µg/ml DCIP in distilled water.

Procedure:

1. Prepare 25% homogenate of fresh Spinach leaves in potassium phosphate buffer with 0.3M sucrose, filter and centrifuge the filtrate at 3000 rpm for 5 min.
2. Re-suspend the chloroplast pellet in 10 ml of buffer and store on ice. In a test tube take 1.9 ml water, 3 ml buffer, 0.5 ml chloroplast suspension and 0. 1 ml DCIP, mix and incubate in sunlight for 1 hour.
3. A suitable control should also be prepared and incubate in dark. Prepare a standard curve using different concentrations of DCIP on X-axis against absorption at 550 nm on Y-axis.

For drawing Standard Graph please refer figure 1.

Then from this standard curve calculate the amount of DCIP reduced.

Standard Curve:

Sl. No.	Water	DCIP	Concentration of DCIP (µg)	A_{550}
1	5.5	0.0	0	0.00
2	5.3	0.2	6	
3	5.1	0.4	12	
4	4.9	0.6	18	
5	4.7	0.8	24	
6	4.5	1.0	30	

Test:

Serial Number	Water	Buffer	Chloroplasts	DCIP	A_{550} against water
T-1 Incubated in dark	1.9	3	0.5	0.1	
T-2 Incubated in sunlight	1.9	3	0.5	0.1	

Calculation:

Amount of DCIP reduced = O D of T_1 – O D of T_2 = ……..

Report: The given sample showed --- µmole of DCIP reduced per hour.

EXPERIMENT 6

To separate the leaf pigments by paper chromatographic technique.

Theory:

Chromatography is a technique through which the components of a mixture are separated on the basis of color differentiation. Most common methods include paper chromatography, which is two dimensional and various components are identified on the basis of Rf (Retention Factor) values:

REUIREMENTS:

Fresh grass leaves, pestle and mortar, Whatmann filter paper no. 1, glass jar, one holed rubber cork, micropipette, 10 ml measuring jar, acetone and petroleum ether.

PROCEDURE

1. Fresh grass leaves are homogenized in the pestle and mortar with equal amount of petroleum ether and acetone and the extract is prepared.
2. A narrow strip of Whatmann paper no.1 is taken.
3. Near the pointed end, the extract is spotted and it is called as loading spot. The spot is loaded again and again and air dried.
4. The pointed tip of the Whatmann paper is dipped in a glass jar containing solvent mixture of petroleum ether and acetone in the ratio of 9:1.
5. The loading spot should not touch the solvent.

OBSERVATION:

As the solvent ascends the pigments are carried with the solvent. Depending upon the adsorption capacity, the components get separated and form distinct zones. The orange color indicates the carotene which lies near the solvent front followed by yellow color xanthophyll, bluish green chlorophyll-a, and yellowish green chlorophyll-b. The solvent front (SF) is marked and the distance travelled by the pigment is measured by using this, RF value is calculated. Rf value is calculated by using the formula.

RF = Distance travelled by the pigment from the origin spot / Distance travelled by the solvent from the origin spot

S.F. =

Rf value of carotene =

Rf value of xanthophyll =

Rf value of chlorophyll a =

Rf value of chlorophyll b =

INFERENCE

Rf value indicates the differential solubility and differential adsorption characteristic of the leaf pigments.

EXPERIMENT 7

To separate the leaf pigments by thin layer chromatographic technique.

Principle:

Thin layer chromatographic technique (TLC) is based on the principle of adsorption. The stationary phase used in TLC is adsorbents like silica gel coated onto a inert solid support such as glass plate, mobile phase is either single solvent or mixture of solvents based on the chemical nature of sample i.e. polarity . Sample should be dissolve in mobile phase. Silica gel contains some free Si-OH groups these groups form hydrogen bonds or other vanderwaal interactions with the analyte components, thus adsorption takes place. Identification of amino acids in the given mixture is determined by Rf value.

REQUIREMENTS:

Fresh grass leaves, pestle and mortar, chloroform, methanol, micropipette, 10 ml measuring jar, acetone and petroleum ether, slides and coupling jar.

PROCEDURE

1. Fresh grass leaves are homogenized in the pestle and mortar with equal amount of petroleum ether and acetone and the extract is prepared.
2. A uniform suspension of silica gel is prepared by dissolving it in a mixture of chloroform and methanol in the ratio of 2:1.
3. The suspension is stirred well with glass rod.
4. Two slides are dipped into the suspension and slowly drawn out and drained to the excess. The slides are completely dried.
5. The extract is spotted towards the corner of the slide leaving space from the edge.
6. Spot is loaded again and again and dried.
7. The slides are kept immersed in a coupling jar containing a solvent mixture of petroleum ether and acetone in the ratio of 4:1.
8. The setup is kept undisturbed.

OBSERVATION:

As the solvent ascends the pigments are carried with the solvent. Depending upon the adsorption capacity, the components get separated and form distinct zones. The orange color indicates the carotene which lies near the solvent front followed by yellow color xanthophylls, bluish green

chlorophyll-a and yellowish green chlorophyll-b. The solvent front (SF) is marked and the distance traveled by the pigment is measured by using this, RF value is calculated.

RF = Distance travelled by the pigment from the origin spot / Distance travelled by the solvent from the origin spot

S.F. =

Rf value of carotene =

Rf value of xanthophyll =

Rf value of chlorophyll a =

Rf value of chlorophyll b =

INFERENCE

Rf value indicates the differential solubility and differential adsorption characteristic of the leaf pigments.