Practical Manual

B.Sc. I

(Botany)

Under

Star college scheme

Of

Department of Biotechnology
Government of india, New delhi

By

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EXPERIMENT.1

Simple staining techniques

Requirements

24 hours old culture of Bacilus subtillis, Escherichid coli, and Staphylloceccus oureus. Staining solution of methylene blue,Staining tray, Glass slides, Inoculating loop, Bunsen burner/ spirit lamp, Blotting papers,etc..

Procedure

1). Take clean glass slides, wash and dry them. 2) Prepare bacterial smears of all the bacterial cultures following the usual procedure. 3) Keep a slide (heat fixed smear) on the staining tray and apply about 5 drops of a stain (any one of the above) for the designated period. 4). Pour off the stain and wash the smear gently with slowly running tap water. 5) Blot dry the slide using blotting paper (do not wipe the slide) 6) Repeat steps 1-5 for the other two cultures.

Observations.

1). Examine all the three preparations under oil-immersion objective. 2) Make drawings for each organism. 3) On the basis of the microscopic observations, write description of the organisms indicating colour, shape and arrangement of cells in each.

Results

The bacteria stain a deep blue. The Bacillus is rod shaped with clear areas (i.e.endospores) within them S.aureus cells are spherical,, occurring singly, in pairs and irregular clusters, the small rod of E.coli frequently

EXPERIMENT 2

Gram staining technique

Requirements – 24 hours cultures

Gram staining reagent – Crystal violet, Gram's iodine solution.

95 percent ethyl alcohol
Safranin
Staining tray/ clothes pin
Wash bottle of distilled water
Droppers
Inoculating loop
Glass slides
Blotting paper/Absorbent paper
Lens paper
Bunsen burner/spirit lamp
Microscope

Procedure

1. Make thin smears of *Staphylococcus* and *Escherichia* on separate glass slides.
2. Let the smears air dry.
3. Heat fix the smears.
4. Hold the smears using slide rack or clothes pin.
5. Cover each smear with crystal violet for 30 seconds.
6. Wash each slide with distilled water for a few seconds, using wash bottle.
7. Cover each smear with Gram's iodine solution for 60 seconds.
8. Wash off the iodine solution with 95 pler cent ethyl alcohol. Add ethyl alcohol drop by drop, until no more colour flows from the smear. (the gram-positive bacteria are not affected while all gram- neagative bacteria are completely decolorized).
9. Wash the slides with distilled water and drain.

10. Apply safranin to smears for 30 seconds (Counter-staining)
11 Wash with distilled water and blot dry with absorbent paper.
12. Let the stained slides air dry
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Obserations

1. Examine the slides microscopically using oli-immersion objective.
2. Identity the gram reaction of both the cultures and classify them.
3. Make sketches for morphology of the cultures.
4. Describe the morphology and arrangement of the cells.

Results

Those bacteria that appear purple are referred to as Gram-positive, those appearing pink are described Gram-negative.

In **S. *aureus*** the appear dark purple or blue in colour, thus it is a gram-positive bacterium whereas in E.coli the rods appear pink and is thus a gram-negative bacterium. To get reliable results, one should use cultures that are 18 to 24 hours old.

EXPERIMENT 3

Preparation of Plant Tissue culture media

Requirements.

Constituents of the MS medium.
Erlenmeyer flasks (100,250,500 ml, 1 litre capacity)
Measuring cylinders (100,1000 ml capacity)
Pipettes (1,5,10 ml)
Distilled or demineralized water
pH Meter
1.0 N NaOH, HCL or KOH
Autoclave

Procedure

1. Prepare macronutrients solution in 100 ml distilled water.
2. Prepare micronutrients solution in 100 ml distilled water following the stock solution dilution chart.
3. Add macronutrient and micronutrient solution while stirring into 700 ml distilled water taken in 1 litre Erlenmeyer flask.
4. Add the other heat stable constituents (e.g. sucrose, vitamins and hormones) and agar powder (if desired at a concentration of 0.8 – 1.0 %) (vitamins and auxins can be added after autoclaving for better results).
5. Make the final volume of the medium by the addition of more distilled water.
6. Adjust pH of the medium to 5.7, while stirring, using 0.1 N NaOH or 0.1 N HCL.
7. If solid medium is desired agar is used, heat the solution while stirring until agar is dissolved.
8. Pour the medium into the desired culture vessels (15 ml in a 25 x150mm culture tube and 50 ml n a 250ml flask).
9. Plug the culture vessels with non-absorbent cotton wool wrapped in cheese-cloth, or with any other suitable closure.
10. Transfer the culture vessels to appropriate baskets covered with aluminium jfoil to check wetting of plugs duting autoclaving.
11. Transfer the baskets to autoclave.
12. Sterilize the medium by autoclaving at 121^0c (1.05 kg/cm^2) (15 psi) for the time period depending upon the volume of the medium in the vessel (e.g. for 25,50,100,250,500,1000,2000,4000 ml capacity time required is 20,25,28,31,35,40,48 and 63 minutes respectively).
13. The medium is allowed to cool at room temperature.

Observations and Results

The cooled medium is ready for inoculation and should be stored at 4^0 C for future use.

EXPERIMENT 4

Embryo culture

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Embryo culture, can be defined as the in vitro culture either of the polarized egg, zygote, proembryo or mature embryo.

The applications of embryo culture are:

1. *To obtain rare hybrids*. In many interspecific and intergeneric crosses fertilization occurs normally, but poor or abnormal development of endosperm causes pre-mature death of hybrid embryos. The embryo culture technique is extensively used to rescue such hybrid embryos from wide crosses which otherwise would abort.

2. *To study morphogenesis and nutritional requirements*. It is an ideal technique for studying morphogenesis and nutritional requirements of the developing embryo.

3. *To overcome seed dormancy and fro shortening the breeding cycle of deciduous trees*. The breeding work of deciduous trees is delayed due to long dormancy period of their seeds. By growing excised embryo in culture this period can be reduced.

4. *For raid seed viability test.* The possibility of breaking seed dormancy by embryo culture allows the use of this technique for testing the viability of a particular seed batch rapidly.

5. *Propagation of rare plants.* The seeds of some wild varieties of the cultivated plants do not germinate in nature. Seedlings can be obtained by culturing their excised embryos, eg.seeds of Musa Bulbisiana, a wild relative of commercial banana.

6. *Haploid Production.* Through elimination of chromosomes of one of the parents following distant hybridization, haploids can be produced. For instance in the cross of Hordeum vulgare x H. bulbosum, the chromosomes of H. bulbosum are preperentially lost resulting in haploid embryo plants.

In vivo the embryo follows a definite pattern of cell division and growth. The first divisions usually lead to the production of a linear filamentous structure. From the funicular end of this structure, a globular embryo develops and the remainder forms the suspensor. In dicots, the globular embryo develops into a heart-shaped embryo and finally into the mature torpedo-shaped embryo with well-defined radical and cotyledons.

Depending upon the age of the embryo, the stimulus for its continued growth is thought to be present within its own cells or in the surrounding endosperm. The fertilized egg and proembryo develop on the nutritional resources of the endosperm.

In general, full –grown embryos can be successfully cultured on a standeard mendium containing sugar, mineral salts and vitamins. Younger embryos, in addition to the above, also require trace elements and other growth-promoting substances. Knop's mineral solution was first used for embryo culture but proembryo were unable to develop on it, eg. Capsella bursapastris.

The basic requirements for a successful culture of embryos is a well-balanced medium containgin macro-and micro-nutrients and a carbon source. Supplementing the medium with organic nitrogen like amino acids or casein hydrolysate can enhance the growth of excised

EXPERIMENT 5

Anther culture

Name of Expt. – To demonstrate the technique of anther culture.

Requirements – 9 cm. sterile petridishes, 9cm. non sterile petridishes, 2 forceps.
Dissecting needles (sterile) (2) prepared from soft glass rod
Iron needles(2)
Scalpel with a narrow blade (sterile)
Cm-scale
Parafilm
Spirit lamp
Dissection microscope
Microscope slides, coverslips(10 each)
Incubator or refrigeration unit for chilling buds (preset at 7^0 - 8^0 C)
Growth chamber equipped with fluorescent lighting (1000-1200 lux)
Plastic pots (50 mm diameter) (10) filled with sterile sand: soil(1:1)

b. Culture media. Washing solutions, sterilizing agents and other chemicals

Culture tubes (10) containing 20ml of medum
20-ml liquid medium in petri dishes (10)
Cultural tubes (10) containing 20ml of root-induction medium
B-medium in 5 cm petri dishes (10)
Culture tubes (10) containing 20 ml or R medium
30- ml of mercuric chloride solution (0.01%) with Tween- 20 as a wetting agent in a petri
Dish
Sodium hypochlorite solution (0.8%)
Ethanol (70%) (for sterilization)

Ethanol (80%v/v) in a coupling jar to be used as a dip. Sterile double distilled water

In 250 ml conical flasks (4)

Acetocarmine stain (1%)

Colchicine solution (0.5% w/v)

c. Source tissue.

Flower-buds of Nicotiana tabacum or other plant.

PROCEDURE

1. Anther culture

i) Do not collect the flower-buds from the plants until the laboratory is ready

ii) Collect the flower- buds using a pair of forceps in a non-sterile petri dish and measure the length of each bud using a cm-scale. Select the buds with a corolla length of 21-23 mm. pollen in these flower-budswould usually be in the late uninucleate or early binucleate stage.

iii) Chill the buds at 7^0 to 8^O C for 12 days in a refrigeration unit.

Iv) Surface- sterilize the buds in a petri dish containgin 0.01% solution of $HgCl_2$ (with Tween-20 added as a wetting agent) for 10 minutes

v) Rinse the buds 3-4 times with sterile double distilled water in a sterile air cabinet

vi) Carefully tease, oenn the buds and remove the anthers using forcepas and a dissecting needle.

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Contents

Experiment No. 1
Effect of plant growth regulators

Experiment 1 A: Effect of gibberellins on plant growth

Object: - To demonstrate the effect of gibberellins on plant growth.

Requirements:- Seedlings of pea (*Pisum sativum)* and beans (Phaseolus vulgaris), 100 ppm gibberellic acid in distilled water, acid washed sand.

Prepare five solutions of gibberellic acid (GA) of different concentrations (molecular weight of gibberellic acid is 348) as under:

1. GA 10^{-1} M (0.01 gm molecular weight of GA dissolved in 1ml ethanol and diluted with water to 100ml)
2. GA 10^{-2} M (1ml 10^{-1} M GA diluted with water to 10ml).
3. GA 10^{-3} M (1ml 10^{-2} M GA diluted with water to 10ml).
4. GA 10^{-4} M (1ml 10^{-3} M GA diluted with water to 10ml).
5. GA 10^{-5} M (1ml 10^{-4} M GA diluted with water to 10ml).

Method and observations :- Use genotypically dwarf as well as tall varieties of pea and bean plants.

a) *Duxarf Pea Seedlings* : 1 – 20 seeds of dwarf pea variety are sown in two rows in acid washed neutral sand under relatively controlled conditions in a greenhouses and wait for 14 days.

2. After 14 days, measure the height of all the 20 seedlings and the length of their leaves and internods.

3. Spray the pea plants of one row with 100 ppm gibberellic acid solution and the other row of pea plants with distilled water containing the same amount of gibberellic acid solution.

4. For 4 weeks observe the weekly changes in the characters studied earlier.

5. Make a table of all the results and prepare curve from the mean of 10 plants for the treatment and control separately to show the differences.

b) *Bean Plants*: 1. Take 6-glazed pots containing good garden soil and sow 5 bean seeds in each pot.

2. Place these pots in greenhouse for 2 to 3 weeks.

3. In each pot, retain two healthy plants and remove the remaining plants.
4. Place one drop of each of the following solutions on the tip of the retained plants in the pot i)distilled water (control), ii) GA 10^{-1}M, iii) GA 10^{-2}M, iv) GA 10^{-3} M, v) GA 10^{-4} M).

The first solution, i.e. distilled water (control) should contain ethanol, equal in amount to these used in the preparation of GA solution.
5. Weekly observe the morphological changes related with the growth for 4 weeks and note them in the from of a table.

Result ;- Application of different concentrations of gibberlic acid show much quicker growth of stem and leaves of pea and bean plants.

Experiment 1 B: Influence of IAA on apical dominance

Object : To demonstrate the influence of IAA on apical dominance.
Requirements : Kidney bean plants (3) or any other potted plants, blade or razor, IAA-Ianolin paste (1%).

Method :- 1. Take 3 kidney bean plants of same age and almost identical growth.
2. Remove the apical bud of two plants with blade and leave one plant undisturbed.
3. Apply 1% IAA-lanolin paste at the tip of one of the decapped plant and plain lanolin at the tip of another decapped plant. Observe after about two weeks.

Results : (a) All lateral buds remain inhibited by the shoot tip in the control plant (A). Lateral buds begin to grow in the plant in which plain lanolin was applied (B). Lateral bud growth remains inhibited in the plant on which IAA-lanolin paste was applied. This indicates the influence of IAA on apical dominance.

Experiment 1 C: Effect of Kinetin on cell division.

Object : To show the effect of kinetin on cell division.

Requirements: Freshly cultured tissues of carrot or tobacco in the culture medium, kinetin, petridishes, alcohol, distilled water, beakers, pipettes.

Method : 1 Culture the tissues of carrot or tobacco in six petri-dishes containing culture medium as discussed earlier in the chapter of Tissue Culture.
2. Prepare various concentrations (1 ppm to 5 ppm) of kinetin.
3. Pour 5ml each of 1 ppm kinetin in one petridish. 2 ppm kinetin in 2^{nd} petri-dish, 3 ppm kinetin in 3^{rd} petri-dish, 4 ppm kinetin in 4^{th} petridish and 5 ppm kinetin in 5^{th} petri-dish. Keep the 6^{th} petri-dish as control and add in it only distilled water instead of kinetin. Observe after about a weak.

Observation and Results:

Experiment No. 2

Preparation of Biofertilisers, Biopesticides and Bioherbicides

Blue green algae in open air, shallow culture. Each farmer can prepare his own algal biofertiliser as follows. It has been recommended that trays (6'+3'+9') of galvanized iron, sheet or brick and morter or pits lined with polythene be prepared. A mix of 10kg soil and 200gm of superphosphate is added to trays and water filled in a trays to the level of 2-6 inch. It is necessary to keep the ph of the soil to neutral and *Tolypothrix, Nostoc, Anaebaena*, and *plectonema* is sprinkled is over the water in tray within a week time, a thick allgal slum is formed over the surface. The water is allowed todry at this stage and the dried algal flakes are collected from the trays and stored in polythene bags and applied at the rate of 10kg/ha in the field, one week after rice transplantation.

Rhizobium:

Rhizobium production is based on batch culture technology. For the growth of rhizobia, good aeration is required and an aeration rate of 5 litres air/hour is adequate. Aeration requirement varies from species to species. For fast growing rhizobia, aeration at a rate of 100 liters air/hr for 201 of broth culture for 3 days at 30^0C has been found suitable. Slow growers may need more air depending upon the species. The stuitable temperature for their growth is 28-30^0 C.

The following steps are required in production.

- Isolation
- Authentication and strain selection
- Preparation of mother culture
- Mass production under non-sterile system or sterile system.

Isolation

Suitable culture media are needed for isolation of the required strain. A number of media composition for Rhizobium production hae been recmmmended by different scientists/manufacturers. Rhizobium is heterotrophic bacteria and can readily utilize mono and disaccharides. Mannitol is the traditional carbon and energy source used for small volume cultiavation of all thizobium although some slow growers prefer pentose e.g. arabinose. Most fast growers utilize disaccharides (e.g. sucrose). However, efficient utilization of carbon source depends on aeration, method of sterlisation and particular carbon source.

Some production units do not use calcium carbonate in order to avoid confusion regarding source of turbidity (whether due to bacterial growth of $CaCO_3$). Few reputed orgnisations are using the following composition: 10 ml glycerol, 0.4 g yeast extract, 0.5 g. K_2HPO_4, 0.2 g $MgSO_4$. $7H_2O$ and 0.1 g NaCl per litre of broth and getting successful results.

Process:

Rhizobium forms nodule on the root of legumies. Nodule is the site of nitrogen fixation and Rhizobium is isolated from these as follows.

- Wash roots thoroughly
- Select pink and healthy nodules
- Collect nodule from plants without damaging it
- Immerse undamaged nodule in 95% ethanol for 5-10 seconds.

Experiment : Preparation of Biopesticides

The neem tree is indigenous to India. Indians have revered the neem tree for a very long time. To Millions of Indian, neem has miraculous power. Indian farmers have kept away insects with different neem extracts. The tree is considered so invaluable that it is found in every part of the country, every roadside, every field and almost every house. India has shared its “free tree” and knowledge of its utilization with the world community. The freedom of diverse species to exist and

the freedom of people to exchange knowledge about them are best symbolized in the neem.

After the introduction of chemical agriculture, the use of such neem-based extracts and other products has diminished to a large extent. Farmers have been made more and more dependent on chemical inputs and have lost confidence in their age-old methods. However, the current crop of pests has developed resistance to a wide range of pesticides available. Farmers are thus caught in a vicious circle the moment they start using chemicals.

The Centre for Indian Knowledge Systems (CIKS) has attempted to identify the technologies that farmers were using for pest control before chemical agriculture came into the picture. It has tested these technologies in farmers fields and developed them in such a way that farers can be totally selfreliant wtith these technologies. They need no longer be dependent on the pesticide companies. The preparation of these products is extremely simple, as is their application. In terms of efficiency, these products are very good and, in certain cases, are even more efficient than the commercially available products.

Preparation of extracts

Neem kernel extract:

For 5 liters of water, 1 kg of green neem leaf is required. Since the quantity of leaves required for the preparation of this extract is quite high (nearly 80 kg are required for 1 hectare), this can be used for nursery and kitchen gardens. The leaves are soaked overnight in water. The next day, they are ground and the extract is filtered. The extract is suited for use against leaf eating caterpillars, grubs, locusts and grasshoppers. To the extract, emulsifier is also added.

Remarks: The advantage of using neem leaf extract is that it is available throughout the year. There is no need to boil the extract since boiling reduces the azadirachtin content. Hence the cold extract is more effective. Some farmers prefer to soak the leaves for about one week, but this creates a foul smell.

Neem cake extract:

A hundred grams of neem cake are required for 1 litre of water. The neem cake is put in a muslin pouch and soaked in water overnight. It is then filtered and an

emulsifier is added at the rate of 1 millilitre for 1 litre of water, after which it is ready for spraying.

Neem oil spray:

Thirty milliliters of neem oil are added to the emulsifier and stirred well to ensure that the oil and water can mix well. After this, 1 litre of water is added and stirred well. It is very essential to add the emulsifier with the oil before adding water. It should be used immediately, otherwise oil droplets will start floating. A knapsack sprayer is better for neem oil spraying than a hand sprayer.

Pongam, aloe and neem extract:

One kilogram of pounded pongam cake, 1 kg of pounded neem cake and 250 g of pounded poison nut tree seeds are put in a muslin pouch and soaked overnight in water. In the morning, the pouch is squeezed and the extract is taken out. This is mixed with ½ litre of aloe Vera leaf juice. To this, 15 litres of water are added. This is again mixed with 2-3 litres of cow's urine. Before spraying. 1 litre of this mixture is diluted with 10 litres of water. For an acre, 60-100 litres of spray are used. This is effective in the control of pests of cotton and crossandra.

Custard apple, neem, chilli extract:.

Five hundred millilitres of water are added to 2 kg of ground custard apple leaves and stirred. This is filtered to get the extract and the filtrate is kept aside. Separately, 500 g of dry fruits of chilli are soaked in water over night. The next day, this is ground and the solution filtered to get the extract. One kilogram of crushed neem fruits is soaked in 2 litres of water overnight and the extract is filtered. All the three filtrates are subsequently mixed with 50-60 litres of water, filtered again and sprayed over the crops.

Note: For all the above extracts. 250 milliliters of khadi soap solution should be added as an emulsifier before spraying.

General remarks about spraying:.

a) Spraying should be undertaken in the morning or late in the evening. Under hot conditions, the frequency of spraying should be increased in winter, spraying once in 10 days and every day in the rainy season is recommenede.

b) Insects lay eggs on the underside of the leaves. Hence it is important to spray under the leaves also.

c) while using a power sprayer, the quantity of water used should be halved.

d) It is better to use low concentrations of extracts frequently.

e) As a general guideline, it can be said that each acre of land to be protected can be sprayed with 60 liters of ready-to-use solution (not the concentrate). Of course, the volume may have to be varied depending on the exact conditions prevailing, such as the intensity of the pest attack.

Biological effects of neem on insects:

The action of neem products as pest control agents can be manifested at different levels and in different ways. This is a very important point to be noted since the farmer would be used to the "knock-out" effect of chemical pesticides. Neem extracts do not exhibit this type of effect on pests but affect them in several other ways.

Experiment 3

Effect of Biopesticides and Bioherbicides on plant

Nitrogen fixation which is required for the growth of higher plants is hindered by pesticides in soil. The insecticides DDT,Methyl parathion and especially pentachlorophenol have been shown to interfere with legume rhizobium chemical signaling result in reduced nitrogen fixtion and thus reduced nitrogen crop yield. Root nodule formation in these plant saves. Pesticides have some direct harmful effect on plant including poor root hair development shoot yellowing and reduced plant growth.

Weed killers (herbicides) can save considerable labor in the yard and garden. Some of these kill plants selectively, so the manager can control weeds but not injure desirable plants. Others are not selective and may kill all plants in an area. They must be applied directly to weeds carefully to avoid damaging nearby plants.

Like many powerful tools, weed killers cause serious harm if used incorrectly.

When using an herbicide, or any pesticide read the label. The label will state whether protective clothing needs to be worn when applying the weed killer.

Make sure that any equipment (such as a sprayer) used is free of other pesticide before adding a new one. It is important always to shower after applying herbicides. Wash clothes separaterly that have been worn when spraying. Failure to follow label directions can result in damage to other plants. Or to animals or people. It is also illegal.

Different herbicides work on different types of plants, on different components of plant metabolism, and at different times in plant growth cycles. Some are translocated (taken up into the plant's vascular system), while others affect the plant on contact. Some pose a risk of moving as a vapor to other sites, while others remain in the soil for long periods, injuring crops planted in those locations at a later time. In either case non-larget plants can be damaged.

It can be difficult to determine whether a plant has been damaged by herbicides or other causes, such as insects or disease. Careful examination of the plant is essential before making any decision about plant damage.

Different herbicides affect different plantsystems, resulting in a range of symptoms from discolored or distorted leaves and stem to a lack of seedling emergence. When landscape plants come into contact with herbicides. Major problems can ensue. Effects may be mistaken for indications of insect infestation,disease, nutritional deficiency or environmental disorder.

Experiment 4

Stages in development of embryo sac

L.s. of ovule showing binucleate embryo sac.

Study the slide of longitudinal section of ovule howing binucleate embryo sac.

Observations.

The slide shows following characters.

The ovule is attached to the placenta by a stalk called funicle. The ovule is made of intefuments and the nucellus. Outermost part of the ovule is made of two integuments. Inner to the integuments lies nucellus. A few layers below the nucellar epidermis, binucleate embryo sac is situated. At the top of the sac, three degenerating megaspores can still be scen. The embryo sac has two nuclei, one at each pole, separated by a large vacuole.

L.s. of ovule showing 4 nucleate embryo sac

Study the slide of longitudinal section of ovule showing 4 nucleate embryo sac.

Observations.

The slide shows following characters.

1). The ovule consists of stalk and the body. 2). The body fo the oule is made of integuments and the nucellus. 3). The outer covering of the ovule is made of two integuments. 4). Nucellus is situated inner ot the integuments. 5). A few layers below the nucellar epidermis, 4 nucleate embryo sac is present. 6). The embryo sac shows four nuclei, out of which two are located at the micropylar end and the rest two at the chalazal end. 7). The nuclei at two ends are separated by large vacuole in the centre.

L.S. of ovule showing 8-nucleate poygonum type of embryo sac

Study the slide of longitudinal section of ovule showing 8-nucleate Poygonum type of embryo sac.

Observations

Following characters are observed.

1). The ovule shows stalk and the body. 2). The body consists of integuments, nucellus and the embryo sac. 3). There are two integuments which form the outermost covering of the ovule. 4). A small amount of nucellus is present between the integuments and the embryo sac. 5). Embryo sac is present deep into the tissue of nucellus. 6). Orgnised 8-nucleate Polygonumtype embryo sac has an egg apparatus, two polar nuclei and three antipodals. 7). Egg apparatus is situated at the micropylar end. It consists of centrally placed egg cell with two synergids, one on each side of the egg. 8). An egg cell has a large vacuole towards its micropylar end while synergids have a small vacuole toward its chalazal end. 9). Each synergid has a beak like structure on its lateral side and filiform apparatus at its micropylar end. 10). Two polar nuclei are located in the centre of the embryo sac. These later fuse to form the secondary nucleus. 11). Three antipodal cells are located at the chalazal enc. These degenerate soon, either before or just after fertilization. 12). Since this embryo sac develops from a single megaspore, it is known as monosporic, 8-nucleate Polygonum type embryo sac.

Experiment 5

Vegetative propogation through root, stem and leaf cutting

Types of cutting.

Theoretically, all plants with primary meristems are capable of propagation by means of cutting. Cuttings are generally made from the vegetative parts like root, stem or leaf and even from modified plant part like tubers, rhizomes and bulbs. They may, thus be root cuttings. Stem cuttings, tuber cuttings and so on depending on the part of the organ from which these are made.

Root cuttings: Root cuttings are used for propagation of either succulents where they are capable of producing both the root and shoot primordial or of plants which produce suckers freely under natural conditions. The method has proved auccessful in some plants which could not be raised successfully by other methods. Some of the common plants which are propagated through root cuttings include Cyrilla, Prunus, Albiaaia, Aesculus, Taraxaceum , Chaenomeles , Cladrastis, Clethra, Clerodendron, Rhus, Iles, Loelteutria, Maackla, Rhododendron, Syringla and Robinia.

Stem Cutting: Propagation by stem cuttings is widely practiced by foresters, horticulturists and others. the shoot is cut into segmens of suitable size which are then planted for rooting under suitable conditions. On the basis of the type of wood which is used for making cuttings, they may be hardwood, semihardwood, softwood and hervaceous cuttings.

i) **Hardwood cutting:-** In general, hardwood cutting are used for propagating deciduous trees. They have also been used for propagating plants like olive, citrus and some gymnosperms. Many ornamental plants and furit trees are propagated by hardwood cuttings [10+11] Cuttings are made from the vigorous branches when the tree is still in dormant state and are planted as soon as the active season sets in.

ii) Semi hardwood cutting: The cuttings made from the wood of evergreen broadleaved species or form partially mature wood of deciduous plants are semi-

hardwood and are used for propagating many ornamental and fruit plants. Cuttings are usually made from the upper parts of branches although the lower parts can also root. Cuttings in which some of the upper leaves are retained are then planted for rooting under conditions of minimum water lo: this is achieved by planting them under mist sprays.

iii) Softwood cuttings: these are the cuttins which are made from the saft green succulent new growth and are used for propagating many ornamental woody shrubs. Such cutting root easily and quickly if careis take to retain leaves and they are planted under conditions of minimum water los in green houses under constant mist sprays. The most suitable material maing cuttings is the lateral or side branches of the stock plants. Head back the main shoot is helpful as it causes the production of more shoots f te cuttings to be made from. Some of the fruit and other plants are propagated through softwood cuttings [12-14]

Iv) **Herbaceous cuttings:** Cuttings made mostly from greenhouse plar are herbaceous, soft, tender and succulent and require special attention. The root easily under favourable conditions which are similar to those needed of softwood cuttings. Cuttings of most of the floriculture crops belong to category.

Leaf cuttings: In general, plants possessing thick and fleshy leaves may be propagated by leaf cuttings. Thin textured leaves are not suitable for the purpose as they dry quickly. The leaf cuttings when planted suitably, develop both adventitious roots as well as shoots. A new poant is thus, establishe. The cutting itself does not become a part of the new plant. *Bryophyllui Sansevieria and Begonia* are the common plants theat are propagated through leaf cuttings .

Practical Manual

B.Sc. III

(Botany)

Under

Star college scheme

Of

Department of Biotechnology
Government of india, New delhi

By

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Contents

EXPERIMENT 1

Micropropogation

Aim :- Demonstration of the technique of micropropogation using different explants

Requirements :- The stock plants, incubator, laminar flow cabinet, growth cabinet or glass house, 70% ethanol, manoxol(or any other surfactant), distilled water, 2% chloros, nutrient media MS or B5 (powdered and prepared), compost (3:2, peat : sand), 50mm and 85mm diameter sterile petri dishes, 250ml sterile jars (or conical flask), propagation trays, seed trays, plastic bags, glass sheets, forceps, blades, scissor, needles, scalpel, hand gloves, laboratory sealing film, pots, etc.

Procedure :- the following is a representative schedule for a typical micropropogation. However, the procedure would require modifications and adjustments depending upon the material used and the laboratory facilities available.

1. Cut off the shoot tip with a clean and sharp blade.
2. Put the severed apical meristems (or axillary buds) in distilled water.
3. Conduct the next few steps in laminar flow cabinet or under perfect aseptic conditions.
4. Transfer upto 20 axillary buds in a sterile test tube.
5. Fill the test tube with 70% ethanol. Allow ethanol to remain in the test tube as such for about 2 minutes.
6. Pour alcohol and replace it with 2% chloros solution. Shake the test tube vigorously (about 120 strokes per minute) for about 10 minutes.
7. Decant the chloros solution and fill the test tube with sterile distilled water.
8. Rinse the material for 3 to 4 times. Store it in distilled water (but not for more than 2 hours).
9. Empty the contents of the tube into a sterile petri dish.
10. Pour MS (MS I) medium in a sterilized petri dish. Place up to 4 buds (50mm petri dish) or 10 buds (85mm petri dish).

11. Take care to ensure that each bud is stuck firmly in the medium but is not buried.
12. Seal the petri dish with laboratory sealing film. Make 2-4 fine holes in the film with sterilized needle.
13. Place the petri dish in growth room or incubator. Allow it to remain for a period of about 1 week to 2 months depending upon the material used.
14. If multiplication is desired, cut internodes and transfer apical cuttings to sterile jars containing medium MS 2 or MS 4.
15. The basal portion of the cutting should be firmly placed in the medium without burying it.
16. If plants are desired to be rooted and transferred to compost, instead of steps 14 and 15 follow the procedure given below.
17. Cut off apex with 2 or 3 nodes (about 5mm in length).
18. Transfer about 12 plants per 250ml jar containing culture medium MS 4.
19. Allow them to grow for about 3 to 5 days till approximately 5mm long roots develop.

20. Remove each plantlet carefully without disturbing the roots. Also try to remove from roots as much as agar is possible.
21. Fill polythene bags or small pots with compost. Water the bags or pots as the case may be.
22. Transfer the plantlets to damp compost.
23. Maintain high humid conditions for about 12 to24 hours.
24. Transfer the polythene bags or pots to glass house. Leave them in shade away from the direct sunlight.
25. Once the plants show signs of maturity (about 70mm in height), transfer them to larger pots or nursery beds.

Nutrient Media Useful in Micropropogation by Axillary Bud Culture (amount of ingredients –gl^{-1} or mgl^{-1})

Chemicals	**Medium 1 (M&S)**	**Medium 2 (M&S)**	**Medium 3 (BS)**	**Medium 4 (M&S)**
NH_4NO_3	1.65g	1.65g	-	1.65g
KNO_3	1.9g	1.9g	2.5g	1.9g
$CaCl_2.2H_2O$	0.44g	0.44g	0.15g	0.44g
$MgSO_4.7H_2O$	0.37g	0.37g	0.25g	0.37g
KH_2PO_4	0.17g	0.17g	-	0.17g
$(NH_4)_2SO_4$	-	-	13.4g	-
$NaH_2PO_4.H_2O$	-	-	0.15g	-
FeNa, EDTA	36.7mg	36.7mg	40.0mg	36.7mg
H_3BO_3	6.2mg	6.2mg	3.0mg	6.3mg
$MnSO_4.4H_2O$	22.3mg	22.3mg	10.0mg	22.3mg
$ZnSO_4.7H_2O$	8.6mg	8.6mg	2.0mg	8.6mg
KI	0.83mg	0.83mg	0.75mg	0.83mg
$Na_2MoO_4.2H_20$	0.25mg	0.245mg	0.25mg	0.025mg
$CuSO_4.5H_2O$	0.025mg	0.025mg	0.025mg	0.025mg
$CoCl_2.6H_2O$	0.025mg	0.025mg	0.025mg	025mg
Nicotinic acid	0.50mg	0.50mg	1.0mg	0.50mg
Thiamine HCL	0.10mg	0.10mg	10.0mg	0.10mg
Pyridoxine HCL	0.50mg	0.50mg	1.0mg	0.50mg
Glycine	2.0mg	2.0mg	-	2.0mg
Sucrose	20.0g	20.0g	20.0g	20.0g
Inositol	0.1g	0.1g	0.1g	-
Glutamine	0.1g	0.1g	0.1g	-
BAP (6-benzyl-amino purine)	0.25mg	0.25mg	0.25mg	0.25mg
GA_3 (gibberellic acid)	0.1mg	-	-	-
pH	5.64	5.64	5.64	5.64
Agar (Difco Bacto)	8.0g	8.0g	8.0g	8.0g

EXPERIMENT 2

Root and shoot formation

Aim :- Demonstration of the root and shoot formation

Requirements :- Controlled temperature growth room, dissecting microscope, laminar air flow cabinet, autoclave, hydrochloric acid, Murashige and Skoog's basal medium, naphthalene acetic acid (NAA), 6-benzyl aminopurine (BAP), kinetin, sodium hydroxide, etc.

Procedure :-

The following is a tentative schedule for a typical plant tissue culture. The exact procedure would, however, differ depending upon the material to be used and the laboratory facilities available.

1. Cut off the shoot tip or any desired part with a clean and sharp blade.
2. Place the severed part in a petridish containing distilled water.
3. Conduct the next few steps in laminar air flow cabinet or under perfect aseptic conditions.
4. Cut portion (explant) is dipped into 5% teepol (or any other surface sterilizer) for 5 to 10 minutes for surface sterilization. Wash the explants in sterilized distilled water.
5. The explants is further surface sterilized by immersing in 70% ethanol (V/V) for 40 to 60 seconds followed by 5% sodium hypochlorite (V/V) for 20-30 minutes.
6. The explants is finally washed at least three times in sterilized distilled water.
7. A thin solid section of the explants is now cut by a sterilized scalpel and placed in sterilized petri dish. 5% sodium hypochlorite solution is now added to the petri dish. The section is allowed to remain in this solution for about 5 – 10 minutes.
8. The explants is now repeatedly washed in sterile distilled water.
9. The explants is then placed in another clean and sterilized petri dish.
10. Prepare sterilized conical flasks with nutrient medium and keep them plugged with cotton.

11. The cambium is carefully removed from the sections under total aseptic conditions.
12. The separated cambial tissue is now transferred to the conical flasks containing medium.
13. The flask marked for induction of root should contain low concentration of cytokinin and relatively high concentration of auxins in the medium.
14. The process of incubation involves removal of cotton plug, insertion of explants and replugging under completely aseptic conditions.
15. Allow atleast 4-6 weeks for the callus growth to take place.
16. Callus tissue can now be removed for sub-culture.

EXPERIMENT 3

Isolation of protoplast

Aim :-Isolation of protoplast from different tissues using commercially available enzymes

Three different tissues are used and therefore, requirements and methods for each one of them are described separately.

EXPERIMENT 3 A

Isolation of protoplasts from mesophyll cells of tobacco by simultaneous method

Requirements :- Tobacco leaves, 70%ethanol, 0.5% sodium hypochlorite solution, distilled water, 600m $mol1^{-1}$ mannitol- CPW solution, (CPW – cell protoplast washing medium contains ($mg1^{-1}$): KH_2PO_4 (27.2), KNO_3(101), $CaCl_2$. 2H2O (1480), $MgSO_4$. $7H_2O$ (246), KI (0.16), $CuSO_4.5H_2O$ (0.025), pH (5.8), enzyme 4% cellulose SS, 0.4% macero-enzyme SS, sucrose, parafilm, forceps, petridishes, Pasteur pipette, 60-80 mμ mesh, centrifuge tubes, etc.

Procedure :-

1. Select fully grown leaves from 7-8 week old plants.
2. Surface – sterilize the leaves by first immersing in 70% ethanol for about 30 seconds followed by rinsing in 0.4-0.5% sodium hypochlorite solution for about 30 minutes.
3. Wash the leaves thoroughly with sterile distilled water to remove all the traces of hypochlorite.
4. Peel off the lower epidermis with fine forceps. Cut them into pieces.
5. Place the peeled leaf pieces on a thin layer of 600m $mol1^{-1}$ mannitol- CPW solution in such a way that the peeled surface is in contact with the solution.

Stock solutions for Murashige and Skoog's (MS) medium

Constituents	**Amount (mg^{-1})**
Stock solution I	
NH_4NO_3	33000
KNO_3	38000
$CaCl_2.2H_20$	8800
$MgS0_4.7H_20$	7400
KH_2P0_4	3400
Stock solution II	
KI	166
H_3Bo_3	1240
$MnS0_4.4H_20$	4460
$ZnS0_4.7H_20$	1720
$Na_2Mo0_4.2H_20$	50
$CuS0_4.5H_20$	5
$CoCl_2.6H_20$	5
Stock solution III[b]	
$FeS0_4.7H_20$	5560
Na2.EDTA.2H20	7460
Stock solution IV	
Inositol	20000
Nicotinic acid	100
Pyridoxine HCL	100
Thiamine HCL	100
Glycine	400

To prepare i litre of medium take 50ml of stock 1,5ml of stock II, 5ml of stock III and 5ml of stock IV.

[b]Dissolve FeSO4. 7H2O and Na2.EDTA. 2H2O separately in 450ml distilled water by heating and constant stirring. Mix the two solutions, adjust the pH to 5.5 and add distilled water to make up the final volume to one litre.

6. After about 30 minutes replace the mannitol – CPW solution by filter-sterilized solution of enzyme containing 4% cellulose SS, 600 m $mol1^{-1}$ mannitol and CPW salts.

7. Seal the petri dish with parafilm and incubate it in the dark at 24-26^{0}C for 16-18 hours.
8. Gently squeeze the leaf pieces with a Pasteur pipette to liberate the protoplasts.
9. Remove the large debris by filtering through 60-80µm mesh.
10. Transfer the filtrate to a screw-cap centrifuge tube and sediment the protoplasts at 100g for 3 minutes.
11. Remove the supernatant and transfer the sediment on the top of 860m mol1-1 sucrose solution (prepared in CPW) in a screw-cap centrifuge tube and centrifuge it at 100g for 10 minutes.
12. Collect the green protoplast band from the top of the sucrose solution and transfer it to another centrifuge tube.
13. Add the protoplast culture medium to suspend the protoplasts and centrifuge at 100g for 3 minutes. Repeat these washings for at least three times.
14. After the final washing add sufficient amount of culture medium to achieve protoplast density of 0.5×10^5 to 1×10^5 ml^{-1}.
15. Plate the protoplasts as small (100-150µl) droplets or a thin layer in petri dishes.

EXPERIMENT 3 B

Isolation of protoplasts from mesophyll cells of Cereals

Requirements:- Wheat/Rice seedlings, 0.1% zephirin, 10% ethanol, washing solution (600m mol1^{-1} sorbitol solution containing 10m mol^{-1} $CaCl^2$), 0.5% macerozyme, 1% hemicelluloses, 2% cellulysin, 600m mol1^{-1} sorbitol, pH 5.4, nylon mesh (pore size 0.7mm- 0.05mm), petri dishes, test tubes, etc.

Procedure:-

1. Take primary leaves from 5-6 days old seedlings by cutting at the base of the leaf. Discard the apical 0.5cm region.

2. Surface – sterilize the leaves in 0.1% Zephirin – 10% ethanol solution for 5 minutes.
3. Wash twice with washing solution (600m mol1^{-1} sorbitol solution containing 10m mol1^{-1} $CaCl_2$).
4. Cut the leaves into 1 – 2mm wide transverse sections and transfer them to the enzyme solution (0.5% maceroenzyme, 1% hemi-cellulose, 2% cellulysin, 600m mol1^{-1} sorbitol, pH 5.4) with a ratio of 10ml enzyme solution for each gram of leaf tissue.
5. Infiltrate the leaves under partial vaccum for 3 – 5 minutes.
6. Incubate the leaves in the dark at 23^{+}-2^0C, on a shaker with 80 strokes per minute.
7. After 2 hours filter the leaf digest through two layers of nylon mesh (first mesh of pore size 0.7mm and the second of 0.05mm).
8. Transfer the filtrate to centrifuge tubes and spin at 50g for 90 seconds.
9. Remove the supernatant and wash the pellet thrice with washing medium.
10. Suspend the protoplast in nutrient medium and culture them.

EXPERIMENT 3 C

Isolation of protoplasts from root nodules

[III] Isolation of protoplasts from root nodules of *Trifolium* sp.

Requirements :- Roots of Trifolium, protoplast dilution buffer (PDB), 4% cellulysin, 2% macerase, 1% driselase, 50µm nylon mesh, Pasteur pipette, 30% sucrose solution

Procedure:-

1. Cut the nodules (1.5mm in length) from aseptically grown plants.

2. Wash thrice with protoplast dilution buffer.
3. Cut the nodules into four pieces and wash again in PDB.
4. Transfer the nodule pieces to the enzyme solution containing 4% cellulysin, 2% macerase, 1% driselase in PDB, pH 5.8.
5. After incubation of about 3 – 4 hours at 23^0C in the dark dissociate the partially digested nodues by passing through the orifice of Pasteur pipette and continue incubation for a further 90 minutes.
6. Sieve through a 50µm nylon mesh and wash the digested tissue twice in PDB by centrifuging at 200g for 10 minutes.
7. Transfer the suspension onto a 30% sucrose solution and centrifuge at 100g for 10 minutes.
8. Collect the intact protoplast from the top of the sucrose solution.

EXPERIMENT 4

Protoplast production

Aim :- Demonstration of protoplast production

Growth and Division of Protoplast

For the growth and nuclear division, the regeneration of cell wall is not seems to be a prerequisite in protoplast culture. In *Convolvulus* sp. Protoplast undergo one or two nuclear division prior to cytokinesis. The same was observed in Haplopappus sp.

It has been observed that for the rapid growth ofprotoplasts frequent subculturing in mannitol free medium is necessary. Protoplasts do not grow in large colonies on high osmotic medium. Its growth gradually shows a downward trend which ultimately inhibited altogether. The colonies, therefore, should be picked up alng with small pieces of agar and then transferred on the top of another medium to avoid any damage to protoplast by rough handling with the forceps.

Protoplast culture : Regeneration of Cell Wall

In culture, protoplasts start developing a wall around itself within few hours and it takes only few days to complete the process. Wall materials are progressively deposited. Cellulose is deposited either between the plasmalemma and the multilamellar wall material or directly on the plamalemma. The nature of biosynthesis of the cell wall depends on the plant material and the system of protoplast culture.

The newly built cell wall can be observed either by plasmolyzing the protoplast by transferring it in a hypertonic solution or by staining the cell wall with *calcofluor*

white fluorescent stain (0.1%). However, electron microscopic studies and freeze etching studies have revealed much about the structure and progressive development of cell wall around the protoplast in culture medium.

Name of some of the families and species in which shoot differentiation of plant regeneration has been achieved from cultured protoplasts.

Family	Species
Compositae	*Cichorium intybus, Lactuca sativa cultivars* (L serriola, L. *saligna*), *Petasites japonicas, Senecio vulgaris*
Cruciferae	*Arabidopsis thaliana, Brassica compestris* (*B. Carinata, B. Juncea, R. Napus, B. Nigra, B. Oleraceae var. capitata*), *Sinapix alba*
Cucurbitaceae	*Cucumis sativus*
Euphorbiaceae	*Manihot esculentus*
Gramineae	*Bromus inermis, Oryza sativa, Pennisetum americanum, Saccharum* spp., *Triticum aestivum*
Leguminosae	*Glycine argyrea, G. canescens, G. clandestine G.max, Medicago arborea, M. Difalcata, Psophocarpus tetragonolobus, Trifolium hybridum, T. Repens*
Liliaceae	*Asparagus officinalis, Hemerocallis* sp
Linaceae	*Linum usitatissimum, L. Strictum, L. Lewissii*
Magnoliaceae	*Liriodendron tulipfera*
Ranunculaceae	Ranunculus sceleratus
Rutaceae	Citrus aurantifolia, C. Grandis, C. Limon
Salicaceae	Populus tremula, P. Alba, P. Grandidentata, P. Nigra
Santalaceae	Santalum album

Solanaceae	*Atropa belladonna, Capsicum annum, Datura metel, D. Innoxia, S. Nigrum, S. Luteum, S. Viarum, S. Xanthocarpum*
Ulmaceae	Ulmus spp.

1. Observe regularly the regeneration of cell wall, cell division and small callus formation under inverted microscope.
2. Examine cell wall formation in protplasts with a droplet of 0.1% calcofluor white R, American Cyanamid, Bound Brooke, N.J. USA, in 0.4m sorbitol solution on a slide. The cell wall regenerated protoplasts fluoresce.
3. Small cluster of calli are observed after 2-3 weeks of culturing protoplasts.
4. Subculture the cell clusters on a freshly prepeared protoplast culture medium with or without ½ the initial mannitol and 0.8-1.6% agar.

Determination of Protoplast Plating Efficiency

Calculate the average number of cell colonies per cm^2 of the petri dish by using following formula:

$$\text{Plating efficiency} = \frac{X \times \text{Area of petri dish} \times 100}{\text{No of protoplast plated/petri dish}}$$

$$X = \frac{S1+S2+S3+S4+S5}{5}$$

Development of Callus/ Regeneration of Whole Plant

Soon after the formation of wall around the protoplasts, the reconstituted cells show considerable increase in size and first divisions give rise to small cell colonies. After 2-3 weeks macroscopic colonies are formed which can be transferred to an osmotic free medium to develop a callus. The callus may

be induced to undergo organogenic differentiaton or whole plant regeneration through embryogenesis.

Organogenesis

Organogenesis takes place from a callus and not directly from a single cell. When isolated protoplasts are put into culture under appropriate conditions of plant inoculums, medium and environmental factors they go through a set series of events.

1. Wall regeneration
2. Early mitotic division and callus formation
3. Organogenesis

Embryogenesis

Plant tissue in vitro can induce to form somatic haploid embryos. Stewards and co-workers first observed the phenomenon of somatic embryogenesis in carrot. Somatic embryos can be induced in cultural conditions from three different sources:

1. Vegetative cells of mature plants
2. Reproductive tissues other than the zygote
3. Hyocotyls and cotyledons of embryo

EXPERIMENT 5

Estimation of Anthocyanin pigment

Aim :- Estimation of Anthocyanin pigment from different plant materials and preparation of absorption spectra

Requirements :- ethyl alcohol, HCL, acetone, petroleum ether, potassium hydroxide, petri plates, Impatients balsamia flower, reagent bottle, separating funnel, colorimeter,

Principle :- The colorimetry or spectrophotometry is based on the principle of Beer-Lambert law. The coloured samples/solutions absorb complementary calories.

Absorbance = LCL

Where L – molar extraction coefficient.

C – molar concentration.

L – optical path length.

O.D. = Aborbance = $\log \frac{I_0}{I}$

Where, Io – incident light intensity

I – emitted light intensity.

Procedure :- Anthocyanins from *Impatients balsamina* flowers can also be separated. Extract is prepared by grinding the material with 1% HCL in 95% ethyl

alcohol. Transfer in a separating funnel, add equal volume of petroleum ether. Shake the mixture and frequently release the pressure which may develop because of mixing of the contents. Wash the mixture separately with distilled water to remove acetone. Discard lower aqueous acetone layer. Collect the upper layer. This layer contains pigment mixture.

Add 25-30ml of 95% ethyl alcohol in separating funnel, mix by rotation. Allow to separate two fractions. Collect the lower fraction (B) in a beaker and transfer in another separating funnel and retain the upper fraction (A)

Lower fraction (B) contains red, purple, blue colour pigment in ethyl alcohol phase :-

Add 30ml ethyl alcohol. Mix by rotation. Wash by adding 5ml distilled water, rotate. Discard lower phase. Repeat washing with water 5-6 time, until distinct layer are observed.

Upper fraction (A) i.e. petroleum ether phase:-

Add 15 ml of freshly prepared 30% ethyl alcohol KOH solution carefully dowm the inner wall, shake and observe for 10 minutes. Add 30ml of distilled water. It separates upper and lower fractions.

Preparation of absorption of absorption spectra :-

Use various fractions obtained in the above experiments

1. Acetone extract
2. Petroleum ether extract
3. Various fractions

Use of calorimeter :-

1. Measure absorbance for each filter of the colorimeter. Set zero for each filter using specific solvent.
2. Note the O.D. for each filter given in the colorimeter manual.
3. Plot the absorbance against wavelength for the filter and find the peak for the maximum absorbance in a certain wavelength (λnm). The O.D. values for different wavelength will give approximate absorption spectrum for different pigments.

Observation table:-

Sr.No	Wave length (nm)	Red (O.D.)	Purple (O.D.)	Blue (O.D.)

Plot the absorbance graph (Optical Density) against the wave length for the filter and find the peak for the maximum obsorbance (λnm).

Conclusion:-

The absorption spectrum differs for each pigment because these pigments absorb light of different wave length in the visible spectrum in between 380nm to 750nm.