THE NEOTIA UNIVERSITY
ज्ञानम् आत्म प्रदीपाय

Micro-propagation Technologies
Practical Manual
Course code EC-AGL-508, EC-AGP-508

2023

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EXPERIMENT – 1

Identification and use of equipment's in tissue culture Laboratory

Aim: Requirements for Plant Tissue Culture Laboratory.

Laboratory Requirements:

'Plant tissue culture' or in vitro cultivation of plants basic requirements:

- Cultivation should be done under aseptic conditions.
- The isolated plant part should get an appropriate environment which will help to divide the cell andto get an expression of internal potential.

Basic facilities for plant tissue culture operations involving any type of in-vitro procedures mustinclude:

- Washing and storage facilities;
- Media preparation, sterilisation and storage room;
- Transfer area for aseptic manipulations;
- Culture rooms or incubators for maintenance of cultures under controlled conditions of temperature,light and humidity;
- Observation or data collection area;
- Transplantation area.

Washing and Storage Facilities:

An area with large sink (lead lined to resist acids and alkalis) and draining area is necessary with provision for running water, draining-boards or racks and ready access to a de-ionized, distilled anddouble-distilled apparatus. Space should also be available to set up drying ovens, washing machines, plastic or steel buckets for soaking labware, acid or detergent baths, pipette washers, driers and cleaning brushes. For storage ofwashed and dried labware, the laboratory should be provided with dustproof cupboards or storage cabinets.

Media Preparation Room or Space:

This part is the central section of the laboratory where most of the activities are performed i.e., mediapreparation and sterilization of media and glassware's needed for culture. There should be sufficient working bench as well as storage space.

The following items are essential in the room:

- Different types of glassware
- Different kinds of balances
- Required chemicals
- Hot plates and Stirrer
- Water bath
- pH meter
- Autoclave and Hot air oven
- Microwave oven
- Vortex, Shaker
- Centrifuge
- Refrigerator and Freezer
- Storage cabinet (Dust-free)

Transfer Area:

Tissue culture techniques can only be successfully carried out in a very clean laboratory having dryatmosphere with protection against air-borne microorganisms. For this purpose, a sterile dust-free room/cabinet is needed for routine transfer and manipulation work.

The 'laminar air flow cabinet' is the most common accessory used for aseptic manipulations now-a- days. The cabinet may be designed with horizontal air flow or vertical air flow where the air is forced into the cabinet through a bacterial HEPA (High Efficiency Particulate Air) filter. The air flows over the working bench at a constant rate which prevents the particles (microorganisms) from settling on thebench.

Before operation in the laminar air flow cabinet, the interior of the cabinet is sterilized with the ultraviolet (UV) germicidal light and wiping the floor of cabinet with 70% alcohol. Inoculation chamber, a specially designed air tight glass chamber fitted with UV light, may also be used as transferarea.

Culture Room:

Plant tissue cultures should be incubated under conditions of well-controlled temperature, illumination,photoperiod, humidity and air circulation. Incubation culture rooms, commercially available incubator cabinets, large plant growth chambers and walk-in- environmental rooms satisfy these requirements.

Culture rooms are constructed with proper air-conditioning; perforated shelves to support the culture vessels, fitted with fluorescent tubes having a timing device to maintain the photoperiod, black curtainsmay be used to maintain total darkness.

For the suspension cultures, gyratory shakers are used. Air conditioners and heaters are used to maintainthe temperature around 25 ± 2°C and humidity is maintained by uniform forced air-ventilation.

Data Collection Area:

The growth and development of tissues cultured in vitro are generally monitored by observing culturesat regular intervals in the culture room or incubators where they have been maintained under controlledenvironmental conditions.

Arrangement should be there where the observations can be done under aseptic conditions using microscope. Special facilities are required for germplasm conservation i.e.; cryopreservation accessoriesshould be there.

Transplantation Area:

Plants regenerated from in vitro tissue culture are transplanted to soil in pots. The potted plants are ultimately transferred to greenhouse but prior to transfer the tissue culture grown plants are allowed foracclimatization under well humid condition and controlled temperature and under controlled entry of sunlight.

EXPERIMENT – 2

Nutrition media composition

Aim: Preparation of MS nutrient medium.
Materials required: Glasswares, chemicals, pH meter, distilled water, autoclave.

Principle:

The basic nutritional requirements of cultured plant cells as well as plants are very similar. However, thenutritional composition varies according to the cells, tissues, organs and protoplasts and also with respect to particular plant species. The appropriate composition of the medium largely determines the success of the culture. A wide variety of salt mixtures have been reported in various media. A nutrient medium is defined by its mineral salt composition, carbon source, vitamins, growth regulators and otherorganic supplements. When referring to a particular medium, the intention is to identify only the salt composition unless otherwise specified. Any number and concentration of amino acids, vitamins, growth regulators and organic supplements can be added in an infinite variety of compositions to a given salt composition in order to achieve the desired results.

Procedure:

1. Dehydrated M S Basal medium (Murashige and Skoog 1962) is used after dissolving indeionized water, to which agar (0.75%) is added.
2. pH of the media to be adjusted between 5.6-5.8 using 1N HCl and 1 N NaOH.
3. For liquid cultures agar is not added.
4. Approximately, 30 ml of the media is poured in 300 ml capacity glass bottles with polypropalinecap each and autoclaved at 121.6°C and 15 PSI pressure for 16 minutes.

Composition of M S Medium	
Ingredients	**milligrams/litre**
Potassium nitrate	1900.00
Ammonium nitrate	1650.00
Calcium chloride.$2H_2O$	440.00
Magnesium sulphate	180.69
Potassium phosphate monobasic	170.00
Manganese sulphate.H_2O	16.90
Boric acid	6.20

Potassium iodide	0.83
Molybdic acid (sodium salt).$2H_2O$	0.25
Zinc sulphate.$7H_2O$	8.60
Copper sulphate.$5H_2O$	0.025
Cobalt chloride.$6H_2O$	0.025
Ferrous sulphate.$7H_2O$	27.80
EDTA disodium salt.$2H_2O$	37.30
myo - Inositol	100.00
Thiamine hydrochloride	0.10
Pyridoxine hydrochloride	0.50
Nicotinic acid (Free acid)	0.50
Glycine (Free base)	2.00
Sucrose	30000.00
TOTAL gm/litre	34.54

Precautions:

- Regular stirring is to be done while dissolving the agar.
- Media should be dissolved in lower volume of around 800 ml and then volume should be madeup to 1000ml.

EXPERIMENT – 3

Sterilization techniques for media, containers and small instruments

Aim: Sterilization techniques.

Materials Required: Autoclave, spirit lamp, glass bead sterilizer, filters, ethanol, laminar air hood.

Principle and procedure

Wet heat (**Autoclaving)**

The method of choice for sterilization in most labs is autoclaving; using pressurized steam to heat thematerial to be sterilized. Sterilization of plant media and autoclavable plastic wares, can normally be achieved in 15 minutes by autoclaving at 121.6° C and15 psi pressure. This method is also useful forsterilization of glassware, cotton, forceps, scalpels etc.

Dry heat (Flaming, Glass bead sterilization)

Inoculation loop can be sterilized by passing an ethanol dipped loop over the flame for a few seconds. Glass bead sterilization is used to keep the metal instruments such as scalpels, scissors, forceps sterilizedin the laminar air flow. Temperature of the glass bead sterilizer is around 250°C-265°C.

Filtration

Filtration is a great way of quickly sterilizing solutions without heating. Filters, of course, work by passing the solution through a filter with a pore diameter that is too small for microbes to pass through. Filters can be scintered glass funnels made from heat-fused glass particles or, more commonly these days, membrane filters made from cellulose esters. For removal of bacteria, filters with an average porediameter of 0.2um is normally used. But viruses and phage can pass through these filters so filtration is not a good option if these are aconcern.

Solvents

Ethanol is commonly used as a disinfectant, although since isopropanol is a better solvent for fat it is probably a better option. Both work by denaturing proteins through a process that requires water, so they must be diluted to 60-90% in water to be effective. Although ethanol and IPA are good at killingmicrobial cells, they have no effect on spores.

Radiation

UV, x-rays and gamma rays are all types of electromagnetic radiation that have profoundly damaging effects on DNA, so make excellent tools for sterilization. The main difference between them, in terms oftheir effectiveness, is their penetration. UV has limited penetration in air so sterilisation only occurs in afairly small area around the lamp. However, it is relatively safe and is quite useful for sterilising small areas, like laminar flow hoods. X-rays and gamma rays are far more penetrating, which makes them more dangerous but very effective for large scale cold sterilization of plastic items (e.g. syringes) duringmanufacturing.

Laminar air hood

Laminar airflow hoods are used in commercial and research tissue culture settings. A horizontal laminarflow unit is designed to remove particles from the air. Room air is pulled into the unit and pushed through a HEPA (High Efficiency Particulate Air) filter with a uniform velocity of 90 ft/min across the work surface. The air is filtered by a HEPA filter so nothing larger than 0.3 micrometer, which includes bacterial and fungal spores, can pass through. This renders the air

sterile. The positive pressure of the airflow from the unit also discourages any fungal spores or bacteria from entering. Depending on the design of the hood, the filters are located at the back or in the top of the box. A UV lamp is attached on the top and initial sterilization for 15- 20 min is carried out using UV lamps followed by air flow through HEPA filter.

Sterilization Procedure:

Principle:

The culture medium, especially when it contains sugar, will also support the growth of micro-organisms like bacteria, fungi etc. So if they come in contact with medium either in cellular form or in spore form, the micro-organisms grow faster than the higher plant tissue due to their brief life cycle and will kill the tissue. The micro-organisms may come from glass vials, instruments, nutrient medium used for culture and even from plant material itself. Therefore, the surface of plant tissue and all non-living articles including nutrient medium should be sterilized.

Procedure:

(i) Sterilization of non-living Articles:

The routine sterilization procedure of non-living articles such as nutrient medium, glass goods, distilled water, instruments (wrapped with brown paper) is by autoclaving under steam at a pressure of 15lbs./in^2 and a temperature of 120°C for 15 minutes.

Thermolabile compounds are often added in the medium and such medium is sterilized either at room temperature or in cold by passing through bacterial filter.

An alternative method of sterilizing glass goods and instruments is by heating in an oven at 150°C for 3-4 hrs.

It should be noted that when autoclaving screw capped glass vials, care should be taken to ensure that the caps are not closed too tightly so that gases can expand without the risk of explosion.

(ii) Sterilization of Plant Material:

Plant material which is to be cultured, should be surface sterilized to remove the surface borne microorganisms. This procedure is done in front of a laminar air flow or inside the inoculation chamber before the plant material is inoculated onto the culture medium.

(1) Thoroughly washed plant material or ex- plant in tap water is immersed in 5% v/v solution of liquid detergent such as 'Teepol' for 10-15 minutes. Then wash the material thoroughly in tap water and finally in distilled water. This step can be done in the general laboratory. Subsequent steps are done in front of a laminar air flow or the pre-sterilized inoculation chamber.

(2) Dip the explants in 70% ethyl alcohol for 60 seconds.

(3) Immediately transfer the material into an autoclaved jaw bottle and pour 0.1% mercuric chloride ($HgCl_2$) 5-10% Sodium hypochlorite (v/v) solution. Keep them for 10- 15 minutes. During that period, the bottle is frequently swirled for shaking so that all surfaces of plant material come equally in contact with sterilant.

(4) After 10-15 minutes, decant the sterilant and wash the explants thoroughly with several changes of autoclaved distilled water to remove all traces of sterilant.

(5) Then the explants are ready for culture.

EXPERIMENT – 4
Sterilization techniques for explants

Aim: Aseptic manipulation of various explants.

SURFACE STERILIZATION OF EXPLANTS

The first important condition for the successful tissue culture procedures is the maintenance of aseptic condition. Sterilization eliminates microorganism and thus avoids contamination by bacteria and fungi. To maintain an aseptic environment, all culture vessels, media and instruments used in handling tissue, as well as the explant itself is should be surface sterilized. Plant material can be surface sterilized by variety of chemicals. Some commonly used chemicals sterilants are as follows:

1 % sodium hypochlorite (NaClO)**:**

It is generally available with 5 % active chlorine content, so 20 % can be used for normal sterilization.

Calcium hypochlorite Ca(ClO)2:

This comes in the powder form. Generally, 100 ml of Ca(ClO)2 is used. The desired weight of hypochlorite is added in to the water, agitated for 10 min, allowed to settle and the clarified filtered supernatant solution is used for sterilization. The filtrate is used immediately because of deliquescent (take up water) nature. Calcium hypochlorite enters the plant tissue slowly as compared to sodium hypochlorite. The standard concentration used is of the order of 4 to 10 % and the soaking time varies from 5 to 30 min.

Bromine Water:

1to 2% bromine water solution is used for the sterilization purpose.

Mercuric chloride:

It is dissolved in water to create the solution. Concentration of 0.01 to 0.1 % for 2 to 10 min, depending upon the tissue, is used. Mercuric chloride is an extremely toxic substance for plant, so rinsing must be very thorough at least five times.

Alcohol:

70 % alcohol is used for sterilization of plant material by dipping them for a period of 30 secs to 2 min. Generally, alcohol alone is not sufficient to kill all the microorganisms and the plant material after alcohol treatment is treated another chemical sterilant.

Antibiotic

Cefotaxime antibiotic at 50 mg/L concentration in the nutrient medium is generally used to control bacterial infection.

Explants after treatment with sterilants must be thoroughly rinsed with sterile distilled because retention of such toxic chemicals will seriously affect the establishment of culture.

REQUIREMENTS

Reagents & Chemicals:

Tween 20 (liquid detergent)**,** 0.1% $HgCl_2$**,** 70% alcohol**,** sterile distilled water

Glassware

Beakers, sterile petri plates, sterile blades, sterile forceps, muslin cloth

Equipment

Laminar airflow hood, Autoclave

PROCEDURE

1. Wash leaf with tap water to remove soil and dust particles deposited on surface.
2. Transfer the washed leaf into a glass beaker containing tap water; add few drops of liquid detergent –Tween 20.
3. Cover beaker mouth with muslin cloth with the rubber band and keep under running tap water for 1hour to remove any waxy/ oily deposition on leaf surface.
4. Wash it twice with distilled water.
5. Transfer the leaf explant into laminar airflow hood for farther work to avoid contamination.
6. Wash the above leaf with sterile distilled water for thrice each washing should be for 3-4 minutes.
7. Treat it with 0.1% $HgCl_2$ solution for 60 sec.
8. After treating it with disinfectant, wash it with sterile distil water for thrice, each washing should befor 3-4 minutes.
9. Wash with 70% alcohol for 30 seconds to remove water from the surface of the leaf.
10. Transfer the sterile leaf to a sterile petri-plate.
11. Cut the leaf into small pieces of about 1x1 cm with sterile blade.
12. Now the explant is ready for inoculation.

EXPERIMENT – 5

Preparation of stocks and working solution

1. Preparation of Culture Medium:

Principle:

Isolated cell, tissues and organs need nutrients for them in vitro growth and development. So, nutrients are supplied artificially according to the medium formulated by several workers. The main objective of medium preparation is to culture the cell, tissue and organ in vitro.

Procedure:

Media should be prepared with care and the following procedure is recommended.

To make 1litre of MS medium:

(i) Dissolve 30gms cane sugar in 200 ml DDH2O. Mix 1-2gms activated charcoal and filter through filter paper, set inside the Buchner funnel fitted on a special conical flask with small side arm attachment. Filtering is done by using a suction pump.

(ii) Take DDH2O in another flask and add in sequence the appropriate amount of stock solution as follows—

Stock solution of macrosalts	50 ml
Stock solution of microsalts	1 ml
Stock solution of KI	1 ml
Stock solution of Fe-EDTA	5 ml
Stock solution of MS 3 vits	1 ml
Stock solution of Glycine	1 ml
Stock solution of meso-inositol	2 ml

Desired concentrations of auxin and/or cytokinin are added from stock solution according to the formula:

Desired concentration/Stock concentration = amount (ml) of stock solution to be taken for one litre medium.

If the quantity of the medium is less than one litre, then hormones are added using another formula—Required concentration X Volume of medium/Stock concentration X1, 000
= amount (ml) of stock solution to be added.

(iii) Pour filtered sucrose solution and salt, vitamins, amino acid, hormone solution mixture into a one litre measuring cylinder. Make the final volume to one litre with DDH2O. Shake well to mix up uniformly.

(iv) Adjust the pH of the liquid medium 5.6-5.8 with the aid of 0.1(N) HCl or 0.1(N) NaOH. This operation is done using the pH metre.

(v) Add 5% to 8% agar to the liquid medium to make solid medium. Heat to 60°C to dissolve the

agar completely. Otherwise, without adding agar, liquid medium can be used for culture.

(vi) Dispense the culture medium into culture tube (20 ml/tube) or wide mouth conical flask (25-40 ml/flask). Insert non-absorbent cotton plug wrapped with gauge cloth. Cover the plug with the help of brown paper and rubber band.

(vii) Medium is finally sterilized by autoclaving.

EXPERIMENT – 6

Culturing of explants: Shoot tip (Meristem Culture)

Aim: To know the process of meristem or shoot tip culture.

What is Meristem Culture?

Meristem culture is the in vitro culture of a generally shiny special dome-like structure mea-suring less than 0.1 mm in length and only one or two pairs of the youngest leaf primordia, most often excised from the shoot apex.

Principle:

The excised shoot tips and meristem can be cultured aseptically on agar solidified sim-ple nutrient medium or on paper bridges dip-ping into liquid medium and under the appro-priate condition will grow out directly into a small leafy shoot or multiple shoots. Alterna-tively the meristem may form a small callus at its cut case on which a large number of shoot primordia will develop.

These shoot primordia grow out into multiple shoots. Once the shoots have been grown directly from the excised shoot tip or meristem, they can be propagated further by nodal cuttings. This process involves sepa-rating the shoot into small segments each con-taining one node. The axillary bud on each seg-ment will grow out in culture to form yet another shoot.

Protocol:

(1) Remove the young twigs from a healthy plant. Cut the tip (1 cm) portion of the twig.

(2) Surface sterilize the shoot apices by incuba-tion in a sodium hypochlorite solution (1% available chlorine) for 10 minutes. The ex- plants are thoroughly rinsed 4 times in ster-ile distilled water.

(3) Transfer each explants to a sterilized petri dish.

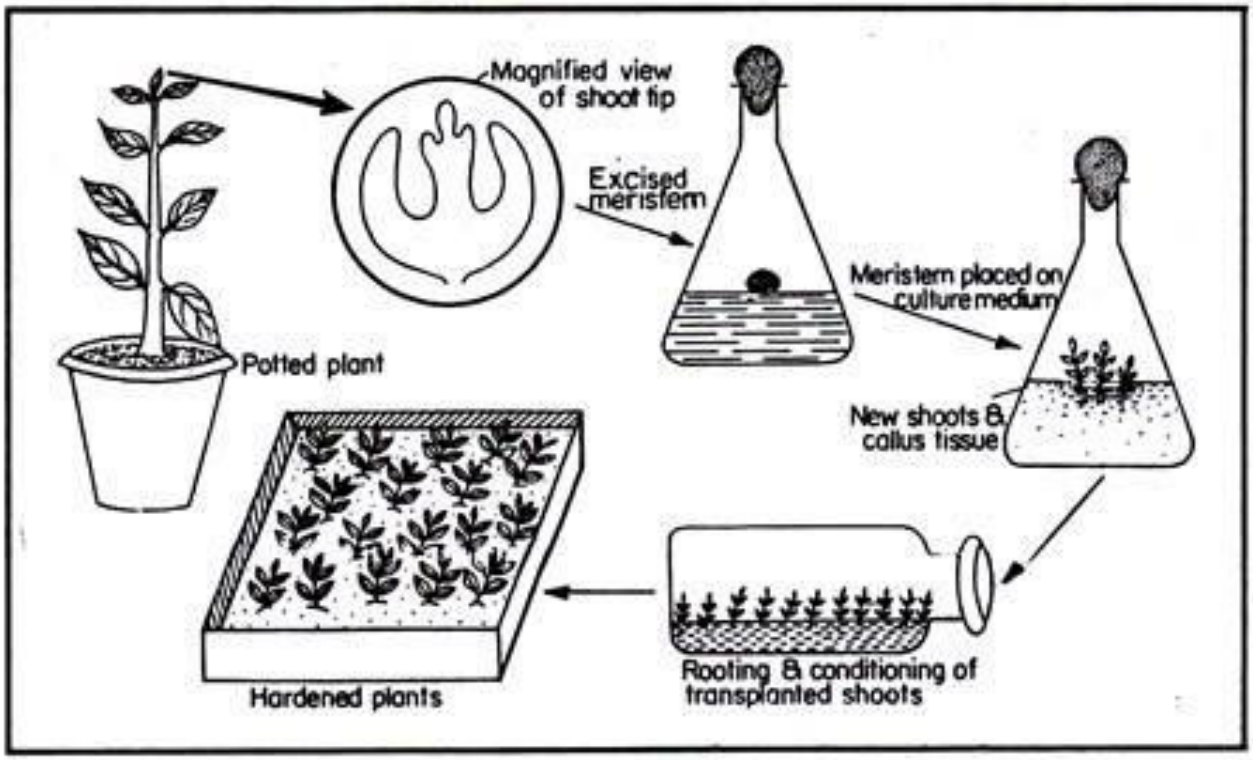
(4) Remove the outer leaves from each shoot apices with a pair of jeweller's forceps. This lessens the possibility of cutting into the softer underlying tissues.

5) After the removal of all outer leaves, the apex is exposed. Cut off the ultimate apex with the help of scalpel and transfer only those less than 1 mm in length to the surface of the agar medium or to the surface of filter-paper Bridge. Flame the neck of the culture tube before and after the transfer of the excised tips. Binocular dissecting microscope can be used for cutting the true meristem or shoot tip perfectly.

(6) Incubate the culture under 16hrs light at 25°C.

(7) As soon as the growing single leafy shoot or multiple shoots obtained from single shoot tip or meristem, develop root, transfer them to hormone free medium.

(8) The plantlets formed by this way are later transferred to pots containing compost and kept under greenhouse conditions.



❒ Fig 2.3

Flow diagram illustrating the technique of shoot tip or meristem culture

EXPERIMENT – 7

Callus induction

Aim: - To induce callus from explant.

Callus is an actively-dividing non-organized mass of undifferentiated and differentiated cells often developing either from injury or in tissue culture in the presence of growth regulators. Explants from both mature and immature organs can be induced to form callus. However, explants with mitotically active cells (young, juvenile cells) are generally good for callus initiation. Callus is producedon explants *invitro* from peripheral layers as a result of wounding and in response to growth regulators, either endogenous or exogenously supplied in the medium. The season of the year, donor conditions of the plant, the age and physiological state of the parent plant contribute to the success of organogenesis in cell cultures. Growth regulator concentration in the culture medium is critical for morphogenesis. Auxin, at a moderate to high concentration, is the primary hormone used to produce callus. In some species, a high concentration of auxin and a low concentration of cytokinin in the medium promotes abundant cell proliferation with the formation of callus. Callus may be serially sub-cultured and grown for extended periods, but its composition and structure may change with time as certain cells are favored by the medium and come to dominate the culture.

Reagents and other requirements

1. Culture tubes or conical flasks containing media
2. Sterile Petri dishes
3. Scalpel, blades, forceps and steel dissecting needles
4. Sterile distilled water
5. Alcohol
6. Detergent (Tween 20, Teepol, etc.)
7. Sterilants – $HgCl_2$, Sodium Hypochlorite
8. Nutrition medium reagents – MS basic salts andvitamins Growth regulators – 2, 4-D

Plant material – Seed Material

Media

Seed Germination: MS Medium
Callus Induction: MS + 2, 4-D (2mg/lL)

I. Seed Germination

1. The seeds washed by submerging in water with a few drops of detergent in a beaker with vigorous shaking.
2. The seeds were submerge in 70% alcohol for 40 s after which the alcohol was decanted.
3. The seeds were transfer to a flask containing 20% commercial sodium hypochlorite solution and left there for 20 min for surface sterilization. Later they were rinsed thrice with sterile distilled water.
4. 2-3 seeds were placed on the surface of MS medium and incubated at 25ºC for 16h photoperiod with 250µE/m^2/ s light intensity for 2 weeks.
5. Observe regularly for germination. If need be, transfer the individual plantlets to half MS medium.

II. Callus Induction

1. The leaves were removed from *in vitro* germinated seeds and were cut into pieces and placed on the MS medium. As a control measure, some explants should be inoculated on MS medium without hormones.
2. The cultures were incubated in dark at 25^{o}C. Callus started appearing within 2 weeks and good callus growth can be observed in 3-4 weeks.
3. Callus can be sub-cultured after the 4th week on fresh medium with the same composition.

Result:

The undifferentiated mass of cells was formed from the inoculated leaf explant.

EXPERIMENT –8

Regeneration of whole plants from different explants

Embryo and Endosperm Culture

Aim: To isolate embryos of *Cicer arietinum* and perform *in vitro* culture

Requirement:

1. Sterilants - alcohol, $HgCl_2$, sodium hypochlorite
2. Nutrition medium reagents - MS basic salts and vitamins
3. Growth regulators – usually not required for embryogenesis
4. Plant Material- Embryo of *Cicer arietinum*
5. Culture tubes containing media
6. Sterile Petri dishes
7. Scalpel, blades, forceps knives and steel-dissecting needles
8. Sterile distilled water

Procedure:

1. The seeds were washed by submerging them in water with a few drops of detergent in a beaker and shake them by hand.
2. The embryo was teased and collected without any damage
3. It was washed with distilled water and then treated with 70% alcohol for 30 seconds.
4. This was followed by rinsing completely with distilled water and then transferred to 20% sodium hypochlorite, where it was left for 0 minutes.
5. Then the embryo was thoroughly rinsed with distilled water for 3 times and dried using the autoclaved tissue paper and inoculated in the culture tubes containing the MS medium.
6. The culture tubes were incubated at 25^{o}C under 16 h photoperiod for 2 to 3 weeks.

Result:

The plant was developed from inoculated embryo.

ANTHER CULTURE

AIM: To isolate and inoculate anthers for haploid production.

PRINCIPLE:

Haploids refer to those plants which possess a gametophytic number of chromosomes in their sporophytes. Haploids may be grouped into two broad categories:

(a) Monoploids which possess half the number of chromosomes from a diploid species.

(b) Polyhaploids which possess half the number of chromosomes from a polyploidy species.

Haploid production through anther culture has been referred to as androgenesis while gynogenesis is the production of haploid plants from ovary or ovule culture where the female gamete or gametophyte is triggered to sporophytic development.

MATERIALS REQUIRED: -

1. Anthers from *Hibiscus*
2. MS medium
3. growth factors
4. 70% ethanol
5. 2% mercuric chloride
6. Meso inositol
7. Scissors
8. Scalples
9. Petriplates
10. Forceps.

PROCEDURE:

1. Flower buds of *Hibiscus* were collected.
2. The flower buds are surface sterilized by immersing in 70% ethanol for 60 sec followed by immersing in 2% sodium hypochlorite solution for 1 min or in mercuric chloride.
3. The buds were washed four or five times with sterile distilled water.
4. The buds were transferred to a sterile Petridish.
5. The buds were split open using a blade and the anther were removed without damage and the filaments were removed.
6. The anther were placed horizontally on the MS medium supplemented with different concentration of plant growth regulators or mesoinositol.
7. The Petriplates were sealed and incubated in dark at 28ºC.
8. The Petriplates were examined for the germination of anthers.

RESULT:

The anther underwent germination leading to the formation of haploid plantlets.

EXPERIMENT – 9

Induction of somatic embryos

SOMATIC EMBRYOGENESIS

Aim: Protocol for somatic embryogenesis in carrot.

Plant Material: Hypocotyl of carrot seedling.

Procedure:

1. Wash seeds by submerging in water with a few drops of detergent in a beaker and shake by hand, or wrap seeds in two layers of cheese cloth/muslin cloth/nylon pouch and then washwith water.
2. Submerge the seeds in 70% alcohol for 30-60s. Decant the alcohol.
3. Transfer the seeds to a flask or beaker containing 20-40% commercial sodiumhypochlorite for 15-20 min. Rinse 4x with sterile distilled water.
4. Place 2-3 seeds per culture vessel on the surface of MS agar medium.
5. Incubate the cultures at 25°C under 16 h photoperiod with -1000 lux light intensity for 1-2 weeks.
6. Collect the germinated seedlings when the cotyledons are fully expanded. Place each seedling on a sterile petri dish and excise the hypocotyl from each seedling and cut them transversely into two parts.
7. Place the hypocotyl sections on the following medium: MS + 1-2 mg/l 2,4-D.
8. Incubate the cultures in dark at 25°C for 4-8 weeks.
9. Maintain the callus by subculturing small pieces on fresh medium every 3- 4 weeks. Callus will contain pro- embryo initial cells as well as minute microscopic embryos in the early stages of development.
10. Place 0.5 to 1 cm^2 callus pieces on MS agar medium without growth regulators andincubate the cultures at 25°C under the 16h photoperiod with ~1000 lux light intensity.Within 2-3 weeks of cultures will exhibit embryos and green plantlets.
11. Tease out individual or group of plantlets from the callus mass and transfer on half strength MS medium under 16h photoperiod with high light intensity of ~5 lux. Within 4-5weeks the cultures will resemble seedling carrots.
12. Transfer the plantlets to small pots containing sterile peat moss and vermiculite in a 1:1 ratio. Enclose the plantlets with plastic containers to maintain high humidity.

13. Transfer the plants to soil and follow the procedure of plant establishment and hardening.

SYNTHETIC SEED PREPARATION

Aim: To prepare hydrated synthetic seeds in *vitro.*

Materials Required:

Beaker
Petri dish
Micropipette
Micro-tips

Chemicals:

Sodium alginate (4%)
Calcium chloride (4%)
Distilled water

Procedure:

1. Embryo was isolated from the viable seeds under aseptic condition.
2. It was transferred to sodium alginate solution and incubated it for 5- 10 mins.
3. The embryo was then transferred from sodium alginate to calcium chloride.
4. The beads formed were transferred to a separate plate for storage.

Result: Hydrated and encapsulated seeds were formed.

EXPERIMENT –10

Hardening procedures.

Aim: Micro-propagation of important crops, hardening and acclimatization of regenerated plants.

Tissue culture is particularly useful for multiplication of plants which are slow growing (turmeric, ginger, cardamom); cross- pollinated (coconut, teak, eucaluptus, cashew, mango and those which show wide variation in the progeny); male-sterile lines (cotton, sorghum, pearl millet); newly produced varieties (normally vegetatively propagated); and for multiplication of virus free plants by meristem cultures (sugarcane, potatoes, tapioca, etc.). Tissue culture is now being commonly used for clonal propagation of a large number of horticultural plants.The success of clonal multiplication in higher plants depends generally on 3 main stages:

STAGE 1: Establishment of an Aseptic Culture

The explants taken from the plant has first to be made free of microorganisms which would outgrow the plant tissue when placed on a nutrient medium. This would result in the death of the explants. These surface contaminants, e.g. bacteria, fungi and yeast are removed by surface sterilization prior to culture, but without killing the plant tissue.

STAGE 2: Multiplication

The surface sterilized material when inoculated on sterile nutrient media and incubated at 25±2ºC with a definite photoperiod and light intensity grows to form large number of shoots.

STAGE 3: Rooting and Hardening of Plants

The shoots obtained are carefully excised and transferred to a rooting medium, preferably a liquid medium, containing an auxin and supported on a filter paper platform in order to obtain rooting in these shoots. These plants which have rooted and have developed secondary roots withroot hairs can be transferred to pots containing soil:vermiculite mixture (1:1). This mixture is pre-autoclaved for 1 hour at 15 psi and steamed for 3 days successively and cooled. The potted plants can be transferred to the field where the first new leaf emerges.

Multiplication by Subculture at Stage 2

However, excised shoot tips can be inoculated on the same medium used in stage 2 instead of therooting media. By regular repetition of this subculture procedure, high rates of multiplication canbe achieved.

Vegetative multiplication of plants depends on various factors as nutrient medium, agar concentration, photoperiod and light intensity, hydrogen ion concentration, size and source of theexplants.

Requirements:

Equipments:

Conical flasks (100ml capacity), Test tubes (25mm*150mm), Petri dishes (80mm diameter), Pair of forceps and scalpel (15 cm long), Environmental growth cabinets adjusted to 25º±2ºC with 18hr photoperiod and 1500lux intensity and15º±2º and 600 lux light intensity. Shaker with 120rpm and 1000 lux light intensity. Culture media, washing solutions, sterilizing agentsGlass distilled water, Sterile glass distilled water0.5%HgCl solution Detergent Medium Source tissue

Procedure:

Sterilization of glassware

Preparation and sterilization of media

Explants collection:

- Select a twig (60-90 cm long, 10-15mm wide) from mature elite trees and cut, making sure that thetwig contains many young axillary buds.
- The length is important in selecting twig that do not wither before being brought to the laboratory.
- Bring the twigs containing axillary bud to the laboratory, remove the leaves and cut them into small pieces of about 5-8 cm.
- Transfer the buds to a sterile 250ml conical flask and surface sterilize the explants.

Culture of buds:

- Keep sterile petri-dishes, scalpel, forceps and medium inside a sterile cabinet along with the flask containing surface-sterilized explants.
- Transfer these explants into sterile petri dishes with the help of a pair of sterile forceps and cut theseexplants into small pieces of 10-15 mm each containing at least one axillary bud.
- Inoculate 2 pieces to each tube containing medium.
- Incubate the tubes in an environment growth cabinet at 15º±2º and 500 lux light intensity for 72 hours.
- Transfer the cultures after 72hr to another incubator maintained at 25º±2ºC with 16hr photoperiod and1500lux intensity.
- After 25 days, the young buds start sprouting.
- When the sprouts are 10-15mm long, transfer them to liquid medium in 100 ml Erlenmeyer flasks.
- Incubate the flasks on a rotatory shaker at 120 rpm and 500 lux light intensity.
- Observe the formation of multiple shoots after 10-15 days.

Multiplication by subculture:

- Transfer the multiple shoots from the flask to a sterile petridish aseptically.
- Incubate the cultures in an environmental growth cabinet at 25º±2ºC and at 1000 lux light intensity (12hr photo periods) and observe the cultures regularly.
- Observe the explants produces multiple shoots within 15 days.
- Separate these shoots again aseptically and transfer the tubes containing medium for shoot formation.

Transfer of plants to pots:

- Remove the rooted plantlet from the tube and wash the roots gently with tap water to remove any tracesof medium.
- Transfer the plantlets to soil: vermiculite (1:1) sterile mixture in a pot.
- Irrigate with about 20 ml of tap water.
- Keep the pots in a growth cabinet at 25º±2ºC and at 1000 lux light intensity and water them.
- Transfer the plants to the field after 8 days of hardening in which 70-80% plants.