Laboratory Manual

Principles of Plant Biotechnology

EXPERIMENT - 1

Aim: Requirements for Plant Tissue Culture Laboratory.

Laboratory Requirements:

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

(a) Cultivation should be done under aseptic conditions.

(b) The isolated plant part should get an appropriate environment which will help to divide the cell and to get an expression of internal potential.

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

(a) Washing and storage facilities;

(b) Media preparation, sterilisation and storage room;

(c) Transfer area for aseptic manipulations;

(d) Culture rooms or incubators for maintenance of cultures under controlled conditions of temperature, light and humidity;

(e) Observation or data collection area;

(f) 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 and double-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 of washed 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., media preparation and sterilisation 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:

(i) Different types of glassware

(ii) Different kinds of balances

(iii) Required chemicals

(iv) Hot plates and Stirrer

(v) Water bath

(vi) pH meter

(vii) Autoclave and Hot air oven

(viii) Microwave oven

(ix) Vortex, Shaker

(x) Centrifuge

(xi) Refrigerator and Freezer

(xii) Storage cabinet (Dust-free)

Transfer Area:

Tissue culture techniques can only be successfully carried out in a very clean laboratory having dry atmosphere 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 the bench.

Before operation in the laminar air flow cabinet, the interior of the cabinet is sterilised 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 transfer area.

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 curtains may be used to maintain total darkness.

For the suspension cultures, gyratory shakers are used. Air conditioners and heaters are used to maintain the 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 cultures at regular intervals in the culture room or incubators where they have been maintained under controlled environmental 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 accessories should 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 for acclimatization under well humid condition and controlled temperature and under controlled entry of sunlight.

EXPERIMENT - 2

Aim: Techniques in Plant Tissue Culture.

The techniques used in plant tissue culture are: (1) Preparation of Culture Medium (2) Sterilization (3) Preparation of Aseptic Plants (4) Aseptic Techniques and (5) Incubation of Culture.

Several techniques have been adopted for in vitro plant cell, tissue and organ culture.

1. Preparation of Culture Medium:

Principle:

Isolated cell, tissues and organs need nutrients for their 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 DDH_2O. 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 DDH_2O 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 DDH_2O. 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.

Technique2. 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 15 lb/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.

Technique 3. Preparation of Aseptic Plants:

Principle:

Surface sterilization of plant tissue may cause some deleterious effect because most of the sterilants are toxic chemicals. Seeds can more or less resist such deleterious effect due to the presence of its seed coat. So to avoid the surface sterilization of plant tissue, seeds are surface sterilized and are cultured on simple basal nutrient medium.

Seeds in culture germinate and give rise to an aseptic seedling. Explants from such seedlings grown under aseptic and controlled conditions are the most suitable material for culture and need no further surface sterilization.

Procedure:

(1) Wash the dry seeds thoroughly with tap water.

(2) Dip the seeds in 5% Teepol solution (v/v) for 10-15 minutes. Decant the Teepol solution and wash the seeds again with tap water and finally with distilled water.

(3) Rinse the seeds with 70% ethyl alcohol for 1 minute.

(4) In front of laminar air flow, transfer the seeds into an autoclaved bottle and pour 0.1% $HgCl_2$ solution (w/v) so that seeds are immersed. Leave for 10-15 minutes. Stir the bottle frequently.

(5) Decant the sterilant and wash 3-4 times with autoclaved distilled water.

(6) Transfer the seeds from bottle to autoclaved petri-dish with the aid of sterile forceps.

(7) Open the closure of the culture vial containing the basal nutrient medium. Flame the neck of the culture vial and in quick succession transfers a few seeds on to the medium. Replace the closure.

(8) Incubate the seeds in continuous dark either at room temperature or at 25-28° C.

Technique 4. Aseptic Techniques:

Principle:

Precautions must be taken to prevent the entry of any microorganism at the time of transferring the surface sterilized explants on the nutrient medium (inoculation) using the sterilized instruments. For this reason, manipulation and transfer should ideally be carried out under aseptic condition. Starting from surface sterilization to inoculation, all operations should be done aseptically.

Procedure:

A typical procedure of aseptic technique is given below:

(1) Put all the sterilized articles (media, instruments, glass goods etc.) for inoculation on the glass racks of the inoculation chamber. Alternatively, if laminar air flow is available, keep all articles on the table of air flow cabinet. Laminar air flow blows bacteria- free air over the working surface.

(2) Put on the switch of UV lamps of inoculation chamber for one hour before work. In case of laminar air flow, the power switch is put on and allows the air flow to blow air for at least 15 minutes before work.

(3) Put off the UV lamp before entering inside the inoculation chamber. Do not put off laminar air flow. The working glass table top of the inoculation chamber or the table of laminar air flow is swabbed with alcohol before starting work.

(4) Wear a clean apron and use a mask. Clean the hands with alcohol and dry it.

(5) Pour alcohol in a clean coupling jar and dip all instruments into it. Light the spirit lamp. Take the surface sterilized or aseptic plant material in a, sterile petri dish.

(6) Flame the neck of culture tube or flask and in quick succession remove the plug of glass vials. Transfer the tissue onto the medium and replace the closure. Each time, the instruments are passed through the flame of the spirit lamp.

Precautions:

(1) Always keep away the hands moistened with alcohol from the spirit lamp. So dry the alcohol first.

(2) Exposure to UV light builds up a high concentration of Ozone gas (toxic) inside the closed chamber. It is, therefore, healthy to enter the chamber only 15-30 minutes after switching off the UV lamp.

(3) Do not dip hot instruments in alcohol and don't use hot instrument for cutting or holding the plant material.

(4) Work carefully and try to ensure that media and plant tissues are exposed for the plant material.

(5) Don't heat the neck of the glass vials excessively.

Technique 5. Incubation of Culture:

Principle:

High temperatures are likely to lead to dissociation of the culture medium and tissue damage while at very low temperatures tissue growth is slow. Again some tissues grow well in dark while others need both light and dark conditions. Low humidity causes the quick desiccation of culture medium and high humidity is favourable for the contamination of culture medium. Therefore, cultures are incubated in a culture room where light, temperature and humidity are controlled.

Procedure:

(1) After inoculating the tissue onto the culture medium, cultures are incubated on culture rack at 25-28°C constant temperature.

(2) Culture tubes are placed at 30-45° inclined position. For this purpose a long wooden stick or an empty paper cover of fluorescence lamp is placed on the middle of culture rack and lay the plugged end of the culture tube on the support.

(3) Illumination is provided by cool-white fluorescent light placed about 18 inches above the culture to give a light intensity of $4 – 10 \times 10^3$ lux for 16 hours.

(4) If light is not necessary, then put off the light and cover the whole rack with a black cloth.

EXPERIMENT - 3

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, the nutritional 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 other organic 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 in deionized 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 polypropaline cap 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 made up to 1000ml.

EXPERIMENT - 4

Aim: Sterilization techniques.

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

Principle and procedure

Wet heat (Autoclaving)

The method of choice for sterilisation in most labs is autoclaving; using pressurised steam to heat the material to be sterilised. Sterilisation 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 for sterilization 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 sterilized in 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 pore diameter 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 a concern.

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 killing microbial 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 of their effectiveness, is their penetration. UV has limited penetration in air so sterilisation only occurs in a fairly 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) during manufacturing.

Laminar air hood

Laminar airflow hoods are used in commercial and research tissue culture settings. A horizontal laminar flow 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 air flow 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.

EXPERIMENT - 5

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 sec 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

Glasswares

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 1 hour 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 be for 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 - 6

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 produced on 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 subcultured and grown for extended periods, but its composition and structure may change with time as certain cells are favoured 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 and vitamins Growth regulators – 2, 4-D

Plant material – Green gram

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 25^oC for 16h photoperiod with $250\mu E/m^2/$ 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 4^{th} week on fresh medium with the same composition.

Result:

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

EXPERIMENT - 7

Aim: Micropropagation 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, pearlmillet); 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 filterpaper platform in order to obtain rooting in these shoots. These plants which have rooted and have developed secondary roots with root hairs can be transferred to pots containing soil:vermiculite mixture (1:1). This mixture is preautoclaved 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 the rooting media. By regular repetition of this subculture procedure, high rates of multiplication can be 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 the explants.

Requirements:

a) Equipments
Conical flasks (100ml capacity)
Test tubes (25mm*150mm)
Petridishes (80mm diameter)
Pair of forceps and scalpel (15 cm long)
Environmental growth cabinets adjusted to 25º±2ºC with 18hr photoperiod and 1500lux intensity and 15º±2º and 600 lux light intensity.
Shaker with 120rpm and 1000 lux light intensity.
b) Culture media, washing solutions, sterilizing agents
Glass distilled water
Sterile glass distilled water
0.5%HgCl solution
Detergent Medium
c) Source tissue

Procedure :-

a. Sterilization of glassware

b. Preparation and sterilization of media

c. Explants collection:

1. Select a twig (60-90 cm long, 10-15mm wide) from mature elite trees and cut, making sure that the twig contains many young axillary buds. The length is important in selecting twig that do not wither before being brought to the laboratory.

2. Bring the twigs containing axillary bud to the laboratory, remove the leaves and cut them into small pieces of about 5-8 cm.

3. Transfer the buds to a sterile 250ml conical flask and surface sterilize the explants.

d. Culture of buds:

1. Keep sterile petri-dishes, scalpel, forceps and medium inside a sterile cabinet along with the flask containing surface-sterilized explants.

2. Transfer these explants into sterile petri dishes with the help of a pair of sterile forceps and cut these explants into small pieces of 10-15 mm each containing atleast one axillary bud.

3. Inoculate 2 pieces to each tube containing medium.

4. Incubate the tubes in an environment growth cabinet at 15º±2º and 500 lux light intensity for 72 hours.

5. Transfer the cultures after 72hr to another incubator maintained at 25º±2ºC with 16hr photoperiod and 1500lux intensity.

6. After 25 days, the young buds start sprouting.

7. When the sprouts are 10-15mm long, transfer them to liquid medium in 100 ml Erlenmeyer flasks.

8. Incubate the flasks on a rotatory shaker at 120 rpm and 500 lux light intensity.

9. Observe the formation of multiple shoots after 10-15 days.

e. Multiplication by subculture:

1. Transfer the multiple shoots from the flask to a sterile petridish aseptically.

2. Incubate the cultures in an environmental growth cabinet at 25º±2ºC and at 1000 lux light intensity (12 hr photo periods) and observe the cultures regularly.

3. Observe the explants produces multiple shoots within 15 days.

4. Separate these shoots again aseptically and transfer the tubes containing medium for shoot formation.

f. Transfer of plants to pots:

1. Remove the rooted plantlet from the tube and wash the roots gently with tap water to remove any traces of medium.

2. Transfer the plantlets to soil: vermiculite (1:1) sterile mixture in a pot.

3. Irrigate with about 20 ml of tap water.

4. Keep the pots in a growth cabinet at 25º±2ºC and at 1000 lux light intensity and water them.

5. Transfer the plants to the field after 8 days of hardening in which 70-80% plants survive.

EXPERIMENT - 8

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^oC 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

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 wash with 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 sodium hypochlorite 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 and incubate 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 4-5 weeks 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

Microtips

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

AIM:- Isolation of Protoplasts.

Protoplast is the living material of the cell where as an isolated protoplast is the cell from which the cell wall is removed. In plant breeding programme many desirable combination of characters could not be transmitted through the conventional method of genetic manipulation.

Plant protoplasts can be isolated from cells by two methods:

1. Mechanical method
2. Enzymatic method.

Mechanical Method

Aim: To isolate protoplast by mechanical method

Principle:

Protoplast can be isolated from almost all plant parts: roots, leaves, fruits, tuber, root nodules, pollen mother cell etc. Protoplast isolated by mechanical is a crude and tedious procedure.Cells are plasmolysed causing the protoplast to shrink from the cell wall. The protoplast obtained from this method is then cultured on suitable culture medium. The principle deficiency of this approach is that the protoplast released is few in number. Mechanical isolation was that of only historical event now.

Materials Required:

1. Plant leaves
2. Mortar and pestle
3. Phosphate buffer pH-7.0
4. 0.3 M sorbitol
5. 0.3 M mannitol
6. Glass slides
7. Microscope.

Procedure:

1. Young leaves were obtained from plants growing outdoors and initially washed with tap water to remove any dust particles.
2. The leaves were washed with phosphate buffer and homogenized gently with the mortar

and pestle.

3. The crude protoplast suspension was centrifuged at very low 50-100 rpm for 10 minutes.
4. The supernatant containing intact protoplast was carefully pipetted out and the pellet containing cell debris and other cell organelles were discarded.
5. Small volume of supernatant was placed in the slides and covered with coverslip.
6. The slide was observed in light microscope to find out viable protoplast

Result: The spherical shaped protoplasts were observed using the microscope.

Enzymatic Method

Aim: To isolate protoplasts by enzymatic method

Principle: Protoplasts are isolated by treating tissues with a mixture of cell wall degrading enzyme in solution, which contain osmotic stabilizer. A most suitable source of protoplasts is mesophyll tissue from fully expanded leaves of young plants or new shoots. The release of protoplast is very much dependent on the nature and composition of enzymes used to digest the cell wall. There are three primary components of the cell wall which have been identified as cellulose, hemicellulase and pectin substance. Pectinase (macrozyme) mainly degrades the middle lamella while cellulose and hemicellulase degrades the cellulose and hemicellulosic components of the cell wall. During this enzymatic treatment, the protoplast obtained should be stabilized because the mechanical barrier of the cell wall which offered support has been broken. For this reason an osmoticm is added which prevents the protoplast from bursting.

Materials Required:

1. Young leaves
2. 70% ethanol
3. 2% cellulose
4. 13% mannitol
5. 0.5% macrozyme
6. CPW salt solution:

KH2PO4 - 27.2mg/l

KNO3 - 101mg/l

CaCl2 - 1480mg/l

MgSo4 - 246mg/l

KI - 0.16mg/l

CaSo4 - 0,026mg/l

pH - 5.8.

PROCEDURE:

1. The young leaves were collected and washed in sterile distilled water thrice.
2. The leaves were cut into small bits.
3. Then the leaves were kept immersed in 13% mannitol for 1 h for pre-plasmolysis.
4. Mannitol was removed after incubation ant sterilized enzyme mixture (Cellulase + macerozyme) was added and incubated at 25ºC in a shaker for 12 h
5. The filtrate was centrifuged at 100g for 5 min to sediment the protoplast.
6. The supernatant was removed and the protoplast pellet was suspended in 10ml of CPW +21% sucrose solution.
7. The mixture was centrifuged at 100g for 5 min. The viable protoplast will float to the surface of the sucrose solution.
8. The supernatant was collected and viewed under microscope.
9. The protoplasts were visualized in microscope.

RESULT: Protoplasts were isolated by enzymatic method and viewed under the microscope.

EXPERIMENT - 11

Aim: Culturing of Protoplast.

Protoplasts culture media generally comprises of nutrients same as those required for regeneration of callus and suspension cultures. However, it requires low concentrations of salts like iron, zinc, ammonium etc. Therefore the best standardised media are B5 and MS with some suitable modification. Increase in the calcium concentration 2- 4 times in protoplast culture medium may be useful in preserving membrane integrity. Energy source (sucrose) in the medium is between 3-5% except in few systems like tobacco where the protoplast culture require lower sugar content (1.5%). Organic nitrogen is introduced in media in the form of CH whereas the concentration of inorganic nitrogen is reduced by lowering the concentration of ammonium nitrate in the regeneration medium. Concentration of vitamins remains same as that used in standard tissue culture media. Both types of growth substances (auxin and cytokinin) are required in different concentrations and combinations for inducing cell wall formation and division of protoplasts. Exact combination of these two types of growth hormones in medium varies according to the species and it has been observed that protoplasts isolated from actively growing cell cultures require high auxin/ kinetin ratio to induce division while those derived from differentiated cells (leaf tissue) may require high kinetin/auxin ratio for regeneration.

Various techniques have been developed for the culture of plant protoplasts:

Multiple Drop Array (MDA) Screening

This technique was developed by Potrykus and co-workers in year 1977, for systematic screening of multiple combinations of media constituents for protoplast culture MDA screening method uses hanging drop technique in which each droplet measuring 40µl represents one combination of factors to be tested as one experimental unit. The droplets are arranged in a regular array of 7x7 drops on the lid of a Petridish (9 cm). Each droplet represents one combination of factors to be tested. To test seven different auxins in combination with four different Cytokinins in the medium, each Auxin or Cytokinin is used in at least seven different concentrations.

Feeder Layer Technique

This technique is a fine approach to culture protoplasts at low density. It was prepared by exposing tobacco cell suspension protoplasts (10 6 cells ml -1) to an X-ray dose of 2x10 3 R , which inhibited cell division but allowed the cells to remain metabolically active . Irradiated protoplasts were then washed thrice and then plated in soft agar medium at a density 2.4 x 10 4 ml-1.On this feeder layer irradiated protoplasts at low density (10-100 protoplasts ml -1) of same species or different species were plated. Protoplasts known for regeneration potential in vitro are generally irradiated for use as feeder layer.

Microdrop Culture

This method has been used initially to culture heterokaryons of Nicotiana glauca (+) and Glycine max and Arabdopsis thaliana (+) Brassica campestris. For this technique specially designed cuprak dishes are required which have smaller outer chamber and a large inner chamber. Inner chamber has numerous numbered wells, each having capacity of 0.25 – 25 µl droplet of nutrient medium. Medium is transferred to the wells of inner chamber with the help of specially designed Drummond pipette. Outer chamber is filled with sterile water to maintain humidity inside the dish, which is then covered with a lid, sealed with parafilm and maintained at optimal light and temperature conditions in culture room. Size of the droplet is a critical factor for division of either single protoplast or heterokaryon as it gives a ratio of cell/volume of culture medium equivalent to its cell density i.e. 2-4x103 ml-1. Increase in the size of droplet would decrease effective plating density.

Co- culture of Protoplasts

Protoplasts from two different species can be cultured together to promote their growth. Metabolically active and dividing protoplasts of two types are mixed in a liquid medium and plated together so that the cross –feeding occurs between the two types. This method provides added advantage to low density protoplasts or that of heterokaryons in helping them to undergo sustained divisions. Co-culture protoplasts should be from different explants so that the calli arising from them could be morphologically distinguished. Mesophyll protoplasts which are green in colour can be grown with protoplasts isolated from cell suspension of albino strain. One can differentiate between green colony formed by mesophyll protoplasts and non - green colonies of albino types.

Other techniques

Electroporation treatment of protoplasts is reported to stimulate division and regeneration in them. Protoplasts suspended in buffer solution (4 times its plating density) if exposed to high voltage(250 – 2000 volt) DC pulses for 10-50 µs after intervals of every 10 seconds could enhance higher DNA synthesis and promote early gene expression for differentiation and regeneration. Heat – shock treatment (45 o C for 5 minutes, followed by 10 s on ice) gives similar stimulatory effect as that of electroporation treatment on protoplast division and regeneration.

EXPERIMENT - 12

Aim: Demonstration on isolation of DNA

Materials Required: Extraction buffer, Microfuge tubes, Mortar and Pestle, Liquid Nitrogen, Absolute Ethanol (ice cold), 70 % Ethanol (ice cold), 7.5 M Ammonium Acetate 65° C water bath Chloroform : Iso Amyl Alcohol (24:1) Water (sterile), TE Buffer (10 mMTris, pH 8, 1 mM EDTA).

Composition of extraction buffer (For 5 ml)

component	Stock concentration	Final Concentration	Volume taken from stock
Tris	1M	0.1 M	500 µl
EDTA	0.5M	20 mM	200 µl
NaCl	5M	1.4 M	1.40 ml

+ CTAB 0.1g (2%) + PVP 0.1g (2%) (Heatat 65° Ctill dissolved)+ β-mercaptoethanol 10µl (0.2%)

Principle:

Isolation of DNA using CTAB exploits that polysaccharides and DNA have different solubilities in CTAB depending on the concentration of sodium chloride. At higher salt concentrations, polysaccharides are insoluble, while at lower concentrations DNA is insoluble. Consequently, by adjusting salt concentration in lysates containing CTAB, polysaccharides and DNA can be differentially precipitated.Polyphenols are compounds that contain more than one phenolic ring (e.g., tannin), a structure that binds very efficiently to DNA. They are naturally occurring in plants, but are also generated when plants have tissue damage (browning). Upon the homogenization of plant tissues, polyphenols are synthesized by liberated polyphenol oxidase. The addition of polyvinyl pyrrolidone prevents the interaction of DNA and phenolic rings by binding up the polyphenols.

Procedure:

1. Weigh 200 mg leaves.

2. Crush with liquid nitrogen.
3. Add 1 ml extraction buffer.
4. Mix well and incubate at 65oC for 30 min.
5. Cool down to room temperature.
6. Add equal volume of chloroform :isoamylalcohol (24:1).
7. Centrifuge at 10000 rpm for 10 min. at 4˚C.
8. Take out upper aqueous phase in fresh tube.
9. Add 0.6 volume of chilled isopropanol.
10. Incubate at -20o C for 1 hour.
11. Centrifuge at 12000 rpm for 15 min. at 4˚C.
12. Discard supernatant and add 1ml 70% ethanol.
13. Centrifuge at 10000 rpm for 10 min. at 4˚C.
14. Discard supernatant and air dry pellet at room temperature.
15. Add 50 µl of TE (10:1) and store at 4˚C for overnight.

Result:

Precautions:

- Material finely ground in liquid nitrogen should be immediately transferred into the extraction buffer.
- In chloroform :isoamyl alcohol extraction, the aqueous phase should be carefully removed and organic phase re-extracted to ensure full recovery of DNA. If no separation is observed between the two phases, may be due to high concentration of DNA and /or cell debris in aqueous phase, dilution with more digestion buffer and re-extraction is the solution.
- Care should be taken to do the operations as gently as possible. Vortexing, pipetting using fine tips etc. should be avoided to prevent the shearing of DNA.
- DNA should not be over dried as resuspension in TE become difficult.
- All the glassware, plastic ware, pestles and mortars etc. should be decontaminated properly. Care should be taken to prevent cross-contamination.
- Blank extraction controls are carried out along with normal extractions to check for any contamination.

EXPERIMENT - 13

Aim:- Demonstration of confirmation of genetic transformation.

Blue-white screening can be used to distinguish between recombinant transformants and non-recombinant transformants. Bacterial colonies are allowed to grow on selective media containing antibiotic and X-gal (5-bromo-4- chloro-indolyl-β-D-galactopyranoside), a colorless chromogenic compound. The plasmid that are engineered for blue-white screening carry a MCS site in between gene that encodes for amino acids for enzyme β- galactosidase which cleaves β-glycosidic bond in D- lactose. X-gal mimic D-lactose and β- galactosidase enzyme acts on X- gal and produces a blue color complex. A successful ligation of the desired gene disrupts the lac Z gene, hence no functional β- galactosidase is produced resulting in white colonies. Hence successful recombinant transformed colonies can be easily identified by its white coloration from unsuccessful blue ones.

Procedure:-

DNA cloning using a plasmid vector: Molecular cloning using a plasmid vector involves five major steps:

(1) Construction of a recombinant DNA molecule.

The vector DNA (the plasmid pUC18) and the foreign DNA insert are cleaved with EcoRI and mixed together in a ligation reaction containing DNA ligase. Plasmid pUC18 carries the ampicillin resistance gene and has a large number of restriction sites comprising a multiple cloning site within a selectable marker gene.

(2) Transfer of ligation reaction products to host bacteria.

Competent E. coli are transformed with ligation reaction products

(3) Multiplication of plasmid DNA molecules.

Within each transformed host bacterium, there is autonomous multiplication of plasmid DNA. Each bacterium may contain as many as 500 copies of pUC18. Some bacteria in the mixture will be untransformed (not carrying either recombinant or non-recombinant plasmid DNA).

(4) Division of host cells and selection of recombinant clones by blue-white screening.

Bacterial cells are plated on a selective agar medium containing the antibiotic ampicillin and X-gal. If foreign DNA is inserted into the multiple cloning site, then the lacZ coding region is disrupted and the N-terminal portion of β-galactosidase is not produced. Since there is no functional β-galactosidase in the bacteria, the substrate X-gal remains colorless, and the bacterial colony containing recombinant plasmid DNA appears white, thus allowing the direct identification of colonies carrying cloned DNA inserts. If there is no insertion of foreign DNA in the multiple cloning site, then the lacZ gene is intact and enzymatically active β-galactosidase is

produced and X-gal is degraded. The bacterial colonies containing non-recombinant plasmid DNA thus appear blue.

(5) Amplification and purification of recombinant plasmid DNA.

A recombinant colony is used to inoculate liquid growth medium. After growing the bacteria overnight, the culture is harvested, bacterial cells are lysed, and the plasmid DNA is purified from other cellular components.

EXPERIMENT - 14

AIM: Agrobacterium tumefaciens-mediated plant transformation.

PRINCIPLE: The pathogenic bacteria Agrobacterium have the capacity to transfer part of itsplasmid DNA (called the T-DNA) into the nuclear genome of plants cells. Two types of Agrobacterium strains are used for plant genetic transformation. In the A. tumefaciens strains, theT-DNA genes encode oncogenes that will induce the formation of a tumor on the infected planttissue. In the A. rhizogenes strains, the T-DNA genes encodeoncogenes that will induce the production ofadventitious roots called the hairyroot tissue. This later is used to produce rapidlychimaericplants with untransformed aerial part and transgenic roots cotransformed with the Ri TDNA and the construct of interest.

The T-DNA transfer to the plant nucleus depends on the expression of the Agrobacterium vir genes that delimit the extent of the DNA sequence transferred to the nucleus, by recognizing specific sequences called T-DNA right and left borders (RB and LB). In between these bordersany DNA sequence can be introduced and transferred into the plant genome. This forms the basis for the generation of transgenic plants.

For this, the oncogenes are deleted from the T-DNA and replaced by selectable marker gene and gene of interest. This T-DNA construct can be placed on another replicon (binary vector) than the vir genes, making the transformation system more versatile. The integration of the T-DNA in the genome probably depends on the plant DNA reparation machinery. Generally one copy of the TDNA is inserted randomly in the plant genome, and gene fusions studies indicated that these insertions preferably occur in transcribed regions or in their vicinity.

The steps involved are:

1. Infection of plant tissues with overnight grown Agrobacterium culture
2. Co-cultivation
3. Post-cocultivation wash and Transient expression assay
4. Culture in selective medium
5. Selection of putative transformed plants
6. Molecular analysis of putative transformed plants

MATERIALS:

- In vitrogerminated seedlings
- A. tumefaciensculture
- Liquid plant growth medium
- Sterilized-petridishes
- Filter discs
- micro-tips

- GUS substrate
- Double distilled water

Procedure:

1. Raise the desired Agrobacterium strain in 20 ml of LB medium with appropriate antibiotics, agitated overnight at 200 rpm at 280 C
2. Concentrate the cells at 5000 rpm for 5 min, resuspend the cells in liquid plant growth medium.
3. Prepare the explants. Submerge the explants in bacterial suspension for 10-20 min.
4. Blot-dry the explants and co cultivate them in tissue culture growth conditions for 2-3 days.
5. Wash the explants with sterile dd water to eliminate Agrobacteria.
6. Incubate few explants in GUS substrate (overnight in the dark at 370C) after for detection of transient GUS expression.

RESULT:

Strong expression of GUS (indigo blue color) was observed in the region of the explants from where the shoots developed. The endogenous GUS activity (color) was not detected in non-transformed (control)explants. GUS activity at the cut ends indicates the susceptibility of explants to Agrobacterium mediated transformation.

EXPERIMENT - 15

Aim: Direct DNA delivery to plant by Particle Bombardment

Principle: The fact that DNA could be delivered into plant cells byphysical means andexpressed in intact cells effectively,revolutionized genetic engineering of plants. Out of the available physical procedures for delivering DNA, particle bombardment is the most preferredmethod as it allows introduction of DNA directly into any plant cell type. With particle bombardment, the difficulties of using fragile protoplasts and host-range limitations associatedwith Agrobacterium are circumvented. The basis of the particle bombardment process is the acceleration of DNA-coated microprojectiles (mainlyparticles of tungsten or gold, with 0.2 to 1.5 μm in diameter) at high speed (about 1500 km/h) towards the living cells. After penetration in thecell, the DNA dissociates fromthe microprojectiles and integrates into the chromosome.

MATERIALS:

- Explants
- Microcarrier (gold particles)
- Plasmid DNA with reporter gene
- Macrocarrier
- Stopping screen
- Macrocarrier launch assembly
- Biolistic Gun

Procedure:

- Soak macrocarriers, holders, stoppingscreens, rupture disks in 95%ethanol for 15 min,

then air dry.

- Coat plasmidsover gold particle and prepare a suspension.
- Drip 6 ~ 10 μl of the suspension on to the macrocarrier.
- Open the valve on the steel cylinder, which contain the pressurized helium,rotatethe

black button (heliumpressure regulator) to adjust the heliumpressure(at least 200 psi

higher than the desired pressure).

- After all the materials are in place, close the chamber door and applyvacuum.
- When appropriate vacuum is reached, activate the fire switch. The gas is held until the

burst pressure of the rupture disk is reached.

RESULT: Strong expression of GUS (indigo blue color) was observed in the bombarded cells of the explants. The endogenous GUS activity(color) was not detected in non-transformed (control) explants i.e, explants bombarded with naked particles. GUS activity in the bombarded cells indicates the direct gene delivery to the target plant cells.

EXPERIMENT - 16

Aim: Demonstration of gel electrophoresis techniques.

Materials required: TAE buffer, Agarose gel (1% in TAE buffer), loading dye, casting tray, gel electrophoresis unit, trans illuminator, ethedium bromide solution, distilled water.

Principle:

Agarose gel electrophoresis is a routinely used method for separating proteins, DNA or RNA. Nucleic acid molecules are size separated by the aid of an electric field where negatively charged molecules migrate toward anode (positive) pole. The migration flow is determined solely by the molecular weight where small weight molecules migrate faster than larger ones.In order to visualize nucleic acid molecules in agarose gels, ethidium bromide or SYBR Green are commonly used dyes. Illumination of the agarose gels with 300-nm UV light (under trans illuminator) is subsequently used for visualizing the stained nucleic acids.

Procedure:

1. Prepare a 1 % solution of agarose by melting 1 g of agarose in 100 mL of 1X TAE buffer in a microwave for approximately 2 min.
2. Allow to cool for a couple of minutes then add 2.5 µl of ethidium bromide, stir to mix.
3. Cast a gel using a supplied tray and comb. Allow the gel to set for a minimum of 20 min at room temperature on a flat surface.
4. Load the following into separate wells - 10 µL 1kb ladder, 5 µL sample + 1µL 6x Loading dye.
5. Run the gel for 30 min at 100 V.
6. Expose the gel to UV light (under transilluminator) and photograph.
7. Confirm DNA quality, presence of a highly resolved high molecular weight band indicates good quality DNA, presence of a smeared band indicates DNA degradation.

Result:

Precautions:

- Temperature of the agarose solution should be around 55-65° C at the time of casting. Avoid pouring it very hot or cold.
- Ethidium bromide is a mutagen and should be handled as a hazardous chemical (Always wear gloves while handling).