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ज्ञानम् आत्म प्रदीपाय

Plant Biotechnology Practical Manual Course No, CC-2021

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Aim: Preparation of plant tissue culture medium

Principle:

The basal medium is formulated so that it provides all of the compounds needed for plant growth and development, including certain compounds that can be made by an intact plant, but not by an isolated piece of plant tissue. The tissue culture medium consists of 95% water, macro- and micronutrients, vitamins, aminoacids, sugars. The nutrients in the media are used by the plant cells as building blocks for the synthesis of organic molecules, or as catalysators in enzymatic reactions. The macronutrients are required in millimolar (mM) quantities while micronutrients are needed in much lower (micromolar, mM) concentrations. Vitamins are organic substances that are parts of enzymes or cofactors for essential metabolic functions. Sugar is essential for *in vitro* growth and development as most plant cultures are unable to photosynthesize effectively for a variety of reasons. Murashige & Skoog (1962) medium (MS) is the most suitable and commonly used basic tissue culture medium for plant regeneration.

Plant growth regulators (PGRs) at a very low concentration (0.1 to 100 mM) regulate the initiation and development of shoots and roots on explants on semisolid or in liquid medium cultures. The auxins and cytokinins are the two most important classes of PGRs used in tissue culture. The relative effects of auxin and cytokinin ratio determine the morphogenesis of cultured tissues.

MATERIALS:

- Amber bottles
- Plastic beakers (100 ml, 500 ml and 1000 ml)
- Measuring cylinders (500 ml)
- Glass beakers (50 ml)
- Disposable syringes (5 ml)
- Disposable syringe filter (0.22 mm)
- Autoclaved eppendorf tubes (2 ml)
- Eppendorf stand
- Benzyl-aminopurine
- Naphthalene acetic acid

INSTRUCTIONS:

MS NUTRIENTS STOCKS

Nutrient salts and vitamins are prepared as stock solutions (20X or 200X concentration of that required in the medium) as specified. The stocks are stored at 4^0 C. The desired amount of concentrated stocks is mixed to prepare 1 liter of medium.

Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15: 473-497

MS major salts	mg/1 L medium	500 ml stock (20X)
1. NH_4NO_3	1650 mg	16.5 gm
2. KNO_3	1900 mg	19 gm
3. $Cacl_2.2H_2O$	440 mg	4.4 gm
4. $MgSO_4.7H_2O$	370 mg	3.7 gm
5. KH_2PO_4	170 mg	1.7 gm

MS minor salts	mg/1 L medium	500 ml stock (200X)
1. H_3BO_3	6.2 mg	620 mg
2. $MnSO_4.4H_2O$	22.3 mg	2230 mg
3. $ZnSO_4.4H_2O$	8.6 mg	860 mg
4. KI	0.83 mg	83 mg
5. $Na_2MoO_4.2H_2O$	0.25 mg	25 mg
6. $CoCl_2.6H_2O$	0.025 mg	2.5 mg
7. $CuSO_4.5H_2O$	0.025 mg	2.5 mg

MS Vitamins	mg/1 L medium	500 ml stock (200X)
1. Thiamine (HCl)	0.1 mg	10 mg
2. Niacine	0.5 mg	50 mg
3. Glycine	2.0 mg	200 mg
4. Pyrodoxine (HCl)	0.5 mg	50 mg

Iron, 500ml Stock (200X)
Dissolve 3.725gm of Na_2EDTA (Ethylenediaminetetra acetic acid, disodium salt) in 250ml dH_2O. Dissolve 2.785gm of $FeSO_4.7H_2O$ in 250 ml dH_2O Boil Na_2EDTA solution and add to it, $FeSO_4$ solution gently by stirring.

PLANT GROWTH REGULATOR STOCK

The heat-labile plant growth regulators are filtered through a bacteria-proof membrane (0.22 mm) filter and added to the autoclaved medium after it has cooled enough (less than 60^0 C). The stocks of plant growth regulators are prepared as mentioned below.

Plant Growth Regulator	**Nature**	**Mol. Wt.**	**Stock (1 mM)**	**Soluble in**
Benzyl aminopurine	Autoclavable	225.2	mg/ ml	1N NaOH
Naphtalene acetic acid	Heat labile	186.2	mg/ ml	Ethanol

The desired amount of plant growth regulators is dissolved as above and the volume is raised with double distilled water. The solutions are passed through disposable syringe filter (0.22 mm). The stocks are stored at -20^0 C.

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.

Aim:- To induce callus from explant.

Principle:

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°C 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°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.

Aim: Micropropagation, 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 15°±2° and 500 lux light intensity for 72 hours.

5. Transfer the cultures after 72hr to another incubator maintained at 25°±2°C 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 25°±2°C 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 25°±2°C 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.

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.

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.