Hands on Training on some of the techniques of Plant Tissue Culture

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Plant tissue culture is the in-vitro aseptic culture of cells, tissues, or whole plants under controlled nutritional and environmental conditions, often to produce clones of plants. The technique primarily relies on plant cells' totipotency which is the capacity of a single cell to express the whole genome during cell division. The ability of cells to change their metabolism, growth, and development is just as significant and essential for the regeneration of the entire plant.

Plant tissue culture technology is being widely used for large-scale plant multiplication. In addition to being used in research, they are now essential for plant propagation, disease eradication, and the generation of secondary metabolites.

Gottlieb Haberlandt, in 1902 tried to cultivate individual palisade cells from leaves in knop's salt solution supplemented with sucrose. The cells sustained for a month stored starch but ultimately did not divide. Despite his failure, he is considered the father of plant tissue culture since his experiment set the stage for developing tissue culture technology. Similarly, Roger J. Gautheret, a French scientist, had encouraging results with culturing cambial tissues of carrots in 1934

Some of the techniques use in plant tissue culture have been selected. These technique involved

1. Preparation of Murashige and Skoog medium (MS medium)
2. Initiation of carrot callus in Murashige and Skoog medium (MS medium)
3. Production of Artificial seed
4. Isolation of protoplast by enzymatic method

Principle for preparation of MS medium

1. Balanced Nutrient Composition: Media should contain essential nutrients in balanced proportions (macro and micronutrients).

2. pH Control: pH should be adjusted to an appropriate level (typically around pH 5.7 for MS) to facilitate nutrient uptake.

3. Growth Regulators: Addition of plant growth regulators like auxins and cytokinins to induce specific growth responses (e.g., rooting, shoot proliferation).

4. Sterility: Media must be sterilized to prevent contamination and ensure the growth of only desired plant tissues.

Materials:

- Macro- and Micronutrients: Salts such as nitrates, phosphates, sulfates, potassium salts, magnesium sulfate, calcium chloride, iron salts (Fe-EDTA), etc.

- Vitamins: Typically, myo-inositol, thiamine (B1), pyridoxine (B6), nicotinic acid (B3), etc.
- Plant Growth Regulators: Commonly used are auxins (e.g., indole-3-acetic acid, IAA; indole-3-butyric acid, IBA) and cytokinins (e.g., kinetin, 6-benzylaminopurine, BAP).
- Agar or Gelrite: Solidifying agents to solidify the medium.
- pH Adjusting Agents: Typically, potassium hydroxide (KOH) or hydrochloric acid (HCl).
- Water: Distilled water for preparing solutions.

1. Appropriate quantities of various stock solution was added in sequence in 200ml of distilled water
2. 30 gms of sucrose was dissolved and the volume was adjusted to 1000 ml by adding distilled water
3. The pH of the medium was adjusted to 5.8
4. 8% agar was added to the liquid medium and the treated to 60° C to dissolve the agar completely
5. 10-12ml of MS medium was poured into culture tubes and plugged with cotton plugs
6. 500 ml conical flask were filled with distilled water and then plug with cotton plug
7. Petriplates containing filter paper were wrapped in aluminium foil
8. The culture tubes, conical flasks containing distilled water, petriplates were all sterilized by autoclaving at 15 psi at 121°C for 10-20 minutes
9. The sterilized glasswares were allowed to cool in a slanting position

Sterilization of the explant

1. Carrot root was clean by scrubbing under running tap water to remove any surface soil.
2. All the diseased and dameged part of fresh carrot were rejected and the skin was scrapped off.
3. Within the laminar air flow chamber, it as thoroughly washed with 70% alcohol for 1 - 2 minutes
4. The explant were then kept in 1% HgCl solution for sterilisation for 2 - 3 minutes
5. After that HgCl was completely remove by washing with sterilized distilled water

Inoculation

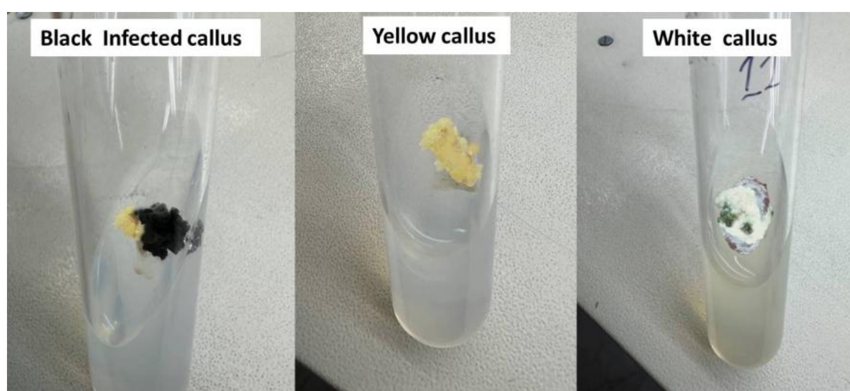
1. Prior to inoculation, all the material were kept in the laminar air flow chamber (except the explant) and UV light was switch on for 45 minutes
2. After 45 munutes, UV light was switch off followed by opening of the hood and switching on of air flow.
3. Hands were sterilised with 70% alcohol. Forceps, scapel were dipped in 70%alcohol and foamed for sterilisation
4. After cooling, explant was cut into thin sections (1.25 mm thick) and inoculated into the nutrient medium
5. The culture tubes were then incubated at 25±2 ºC

Culture tubes inoculated with carrot explant

Students during the inoculation of explant in culture tubes

<u>Observation</u>

The callus tissues vary in appearance and colour and were observed after 21 days. The carrot callus colour observed were yellow and white colour.



Production of Artificial seed in medium (*Cicer arietinum*)

Natural mode of seed production is an ability of most plants . However a novel method to produce an Artificial seed has been tried by man for perfection and betterment in seeds not usually obtain in nature. Thus, Artificial seeds are novel analogous of the natural seeds. The seeds comprises with somatic embryo or shoot bud enclose in hydrogel like sodium or calcium alginate. Most of the sodium alginate produced from algal, that is not toxic is generally used. Thé gel serves as an artificial endosperm during germination if its composition is served as a media. The gel also serves as a seed coat for with standing the environnemental stresses. Artificial seeds is an effective method of propagation of tissue culture raise plants and may even for germplasm conservation.

Requirement:

1. Plant material – embryos of chickpea pea (*Cicer arietinum*)

2. Chemical:

A. Sodium alginate solution - 2.5% of Sodium alginate in 100ml of MS media

B. Calcium chloride solution - 1.472gm of $CaCl_2.2H_2O$ in 100 ml of distilled water.

3. Glasswares: Crucial flask, petri plates, pipette, etc..

4. Magnetic stirrer, pH meter, weighing balance

Procedure :

1. Calcium chloride solution and sodium alginate solution were prepared. Sodium alginate was dissolved slowly with the help of magnetic stirrer.

2. Sodium alginate was poured in a beaker. The plant material were kept in a sodium alginate.

3. Thé plant material along with the sodium alginate were plunged into calcium chloride solution with the help of a spatula while the solution was continuously stirred on a magnetic stirrer.

4. During the formation of beads surface composition begins immediately and ions exchange occur between Na+ and Ça+ forming insoluble sodium/calcium alginate beads result in the formation of artificial seeds

Production of artificial seeds

Isolation of protoplast from *Pisum* sp leaves by enzymatic method .

Isolated protoplast have been described as naked plant cells because the cells is either absent or has been experimentally removed. Proptoplast can be isolated by two methods :

Mechanical Methods : By cutting the cells mechanically

Enzymatic Methods : By using certain enzymes like cellulose, pectinases like macroenzyme or hemicellulose to digest the cell wall. Most often cellulose and macroenzyme are used . The macroenzymes helps in separating the cells so as to release the protoplast . This method gives greater yield . However a suitable osmoticum should be maintained by using mannitol and also so a suitable enzyme concentration is essential so as to achieve proper isolated protoplast without damaging the protoplast. The isolated protoplast has been widely used for somatic hybridisation.

Requirements : Leaves of *Pisum* sp , CPW medium, 70% ethanol, 10% sodium hypochlorite, cellulose, macroenzyme, sterile distilled water, Nylon mesh, centrifuge, sterilized glassware etc

Table : Composition of CPW (Cell and Protoplast Washing) solution used for washing protoplast

CHEMICAL CONSTITUENTS	CONCENTRATION mgL^{-1}
KH_2 PO4	27.2
KNO_3	101.0

$CaCl_2.2H_2O$	14800
$MgSO_4.7H_2O$	246.0
KI	0.16 = 0.08
$CuSO_4.5H_2O$	0.025
pH	5.8

From the CPW solution prepared the following solutions are prepared again :

CPW 13M medium : CPW +13 % mannitol

CPW 9M medium : CPW +9% mannitol

CPW 21S medium : CPW + 21% sucrose

CPW DM medium : CPW salt only as prepared above

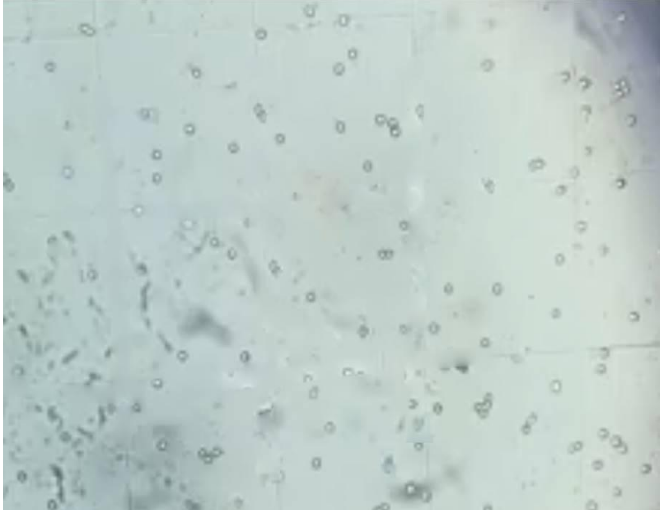
ENZYME SOLUTION : 2% cellulose + 0.5% macroenzyme(13% mannitol+ CPW)

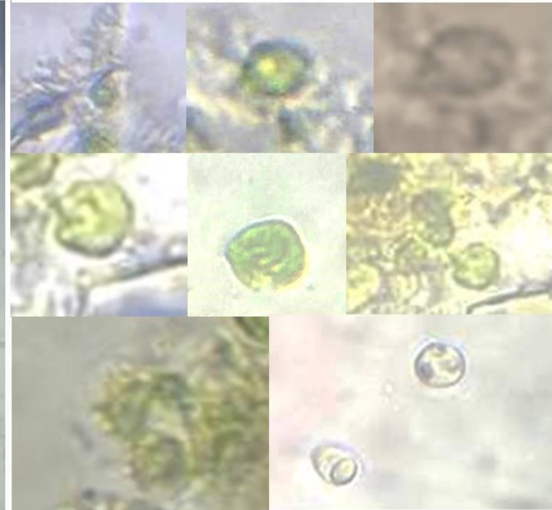
Protocol :

1. CPW solution was prepared and leaves were collected.
2. Incisions were made on the ventral surface and the leaves were chopped off into small pieces.
3. 1gm of the leaves was weighed and washed in 13M CPW (10ml)
4. After washing, the leaves were incubated in the enzymes solution overnight.
5. After the incubation, the enzyme solution containing the protoplast is filtered through the nylon mesh .
6. The filtrate was centrifuged at 100 rpm for 10 mins.
7. The protoplasts settles at the bottom as a pellet and the supernatant was discarded .
8. The pellet was then washed with CPW-9M and centrifuged at 100 rpm for 10 minutes
9. The supernatant was again washed in CPW-21S medium and centrifuged at 100 rpm for 10 minutes
10. The supernatant was collected and observed under the microscope.

OBSERVATIONS:

Spherical protoplasts with numerous chloroplast were observed. They were isolated due to macroenzyme. Numerous fragments of cell debris were also found scattered . Some protoplast bursted due to high osmoticum. Care should be taken to maintain the right osmoticum to get a an intact protoplast





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