CHAPTER 3

GENERAL METHODOLOGY OF PLANT TISSUE CULTURE

The setting up of a tissue culture laboratory needs proper planning. It depends upon space availability, volume of work to be carried out and funds. Usually, the available laboratory space, is be divided into five distinct laboratory areas. These are: *Media preparation area/room, Aseptic transfer chamber area, Environmentally controlled culture room, Analytical room and Acclimatization room.*

MEDIA PREPARATION: AREA/ROOM

Requirements

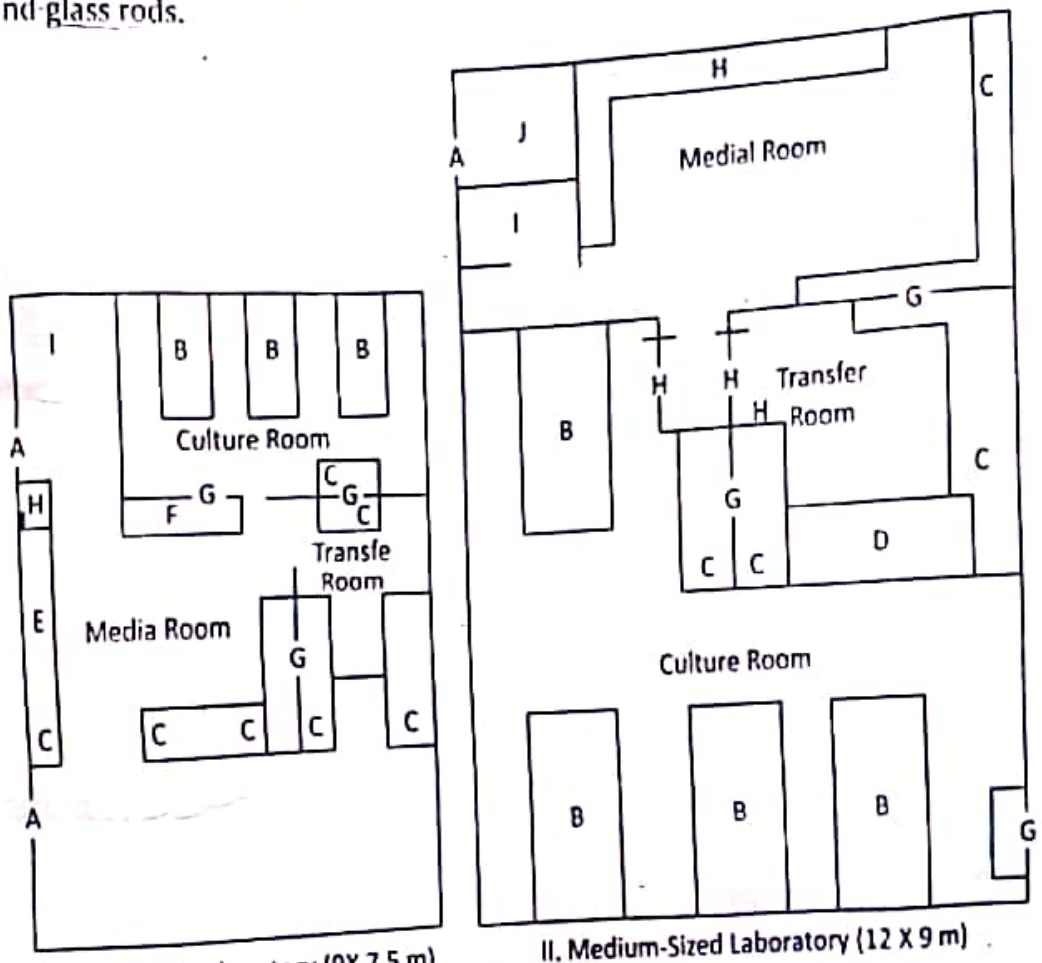
Laboratory tables or benches and revolving stools with adjustable height, Hot plate and magnetic stirrers, analytical loading single pan balance with precision of ± 0.001 g, weighing range 0.1 mg - 180 g, digital read out, Top pan loading balance for quick weighing, range 100 mg - 500 g capacity, sensitivity 0.1 g, Refrigerator and freezer, Water purification and storage system, Glassware washing facility with proper drainage, Gas outlet, Electric hot-air oven range upto 250 ± 2°C and microwave oven, Digital pH meter, Range 0-14 pH, accuracy 0.1 pH temperature compensation 0-100°C, Autoclave preferably horizontal., Continuous supply of single and double distilled water

CABINETS OR SHELVES

For storing glasswares, plastic wares, chemicals, plugs and appliances required for media preparation are as follows:

Requirements

Culture tubes/conical flasks/petri dishes of various capacities, Measuring cylinders 25 ml, 100 ml, 500 ml, 1000 ml, Cotton for plugs/plastic caps (autoclavable), General glassware's/plastic wares of various capacities such as volumetric flasks, beakers, reagent bottles, pipettes, vacuum filtration system and glass rods.



I. Small Sized Laboratory (9X 7.5 m) II. Medium-Sized Laboratory (12 X 9 m)

FIG. 3.1: Various designs of tissue culture laboratory (A) entrance (B) shelves (C) counter (D) laminar air-flow cabinet (E) sink (F) gas outlet/burner (G) window (H) refrigerator and deep freezer (I) store (J) conference room (K) library

CULTURE MEDIA, WASHING POWDER/ LIQUID DETERGENT, DISINFECTANTS

Requirements

Powder or liquid detergents or wetting agents such as Tween-20, 70% alcohol and absolute alcohol. Glass distilled water, Stock solutions of nutrients of tissue culture media or ready-made/pre-mixed powdered media, Sucrose, Agar (tissue culture grade), Sterile culture vessels with distilled water. Chemicals of analytical grades (Inorganic, organic salts, vitamins, amino acids, growth regulators/hormones and activated charcoal), Coconut milk, yeast extract, casein hydrolysate and extracts of potato, carrot and tomato.

Other Requirements

Spatulas, weighing butter paper/boats, stirring bars (magnetic) for magnetic stirrers and stirring retrievers, Brush (flask and test tubes), gloves (disposable 23 cm), mop (household), scoop, towels (household), wastebasket (large and medium), Service lines such as gas, water, electricity, vacuum pump and generator, A low bench/table/desk for culture evaluation and data recording.

ASEPTIC TRANSFER CHAMBER AREA

Requirements

Laminar air-flow cabinets to provide constant flow of air across working area, Dissecting and stereo zoom microscopes and lens magnifier, Micro-dissecting scissors, scalpel handles with blades, forceps (various sizes), catheter trays, needles and inoculating loops, Gas outlet, vacuum facility, Tissue paper/filter paper, Sterilizer (dry heat with glass beads) and pipette dispenser (automatic).

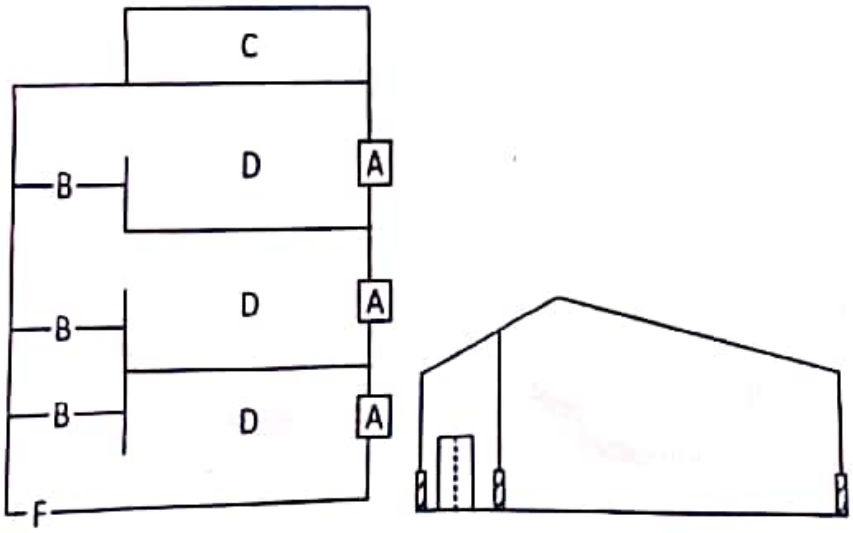


FIG. 3.2: Design and elevation of greenhouse with three compartments having desert coolers (A) Desert-cooler (B) Air-vents (C) Water tank (D) Greenhouse chamber (E) Entrance.

ENVIRONMENTALLY CONTROLLED CULTURE ROOM

Requirements

Racks with light arrangements on timers and controlled temperature (25 ± 2° C) maintained with air cooler or by window air conditioners fitted with temperature indicators, Incubators having dark and light/dark photoperiod with controlled temperature, Rotary shakers of variable speed from 80-220 rpm to take 100 ml or 250 ml Erlenmeyr flasks with arrangement of lighting to provide an intensity of 2000 to 4000 lux, Lux meter to measure intensity of light in culture room, incubator and shaker.

The cultures are usually incubated at 25 ± 2°C under 16:8 light : dark photoperiod. The source of light for the cultures in racks should be make available with cool-day-light (fluorescent tube lights of 40 watts, 2000 lux). It is advisable to connect the controlled culture room with power generator for emergency power supply. Some space should also be kept for incubating cultures in continuous darkness.

ANALYTICAL ROOM

Requirements

Inverted microscope for bright field, dark field with compensating wide angle eye pieces, preferably with photo-micro graphic attachment. Colorimeter for chemical estimation such as chlorophyll, starch, nucleic acid, phenols, oxidizing enzymes etc. Low speed centrifuge with continuous variable electronic speed control, Chemical reagent racks for qualitative and quantitative chemical analysis. Viscosity meter, Gas outlet.

ACCLIMATIZATION ROOM

The hardening chamber needs high illumination (4,000-10,000 lux) and high humidity (90-100%, through mist and fog systems). Humidity is required for conditioning tissue culture plants after taken out from rooting media and transfer to pots under greenhouse.

MISCELLANEOUS ITEMS

Requirements

Air conditioners, uninterrupted power supply (UPS) and emergency light, Bunsen burners, Permanent markers, tapes (autoclave indicator), tape label (self-adhesive), aluminium foil and parafilm, Fluorescent lamps/tubes, Trays and baskets for cultures, Plastic carboys to store water and other solvents, UV germicidal lamp, Metal racks to keep test-tubes in culture room, Gas lighter/match box, Fire saving, equipment and first-aid box etc., Filter paper, culture trays, culture boxes and culture tube racks.

To ensure the growth and development of an explant, it has to be provided with a suitable nutrient medium and proper laboratory conditions for culturing. These operations have to be carried out under aseptic conditions.

SPECIFICATIONS OF LABORATORY EQUIPMENTS

The following laboratory equipments are required for tissue culture.

- **pH Meter (digital):** 230 V 50 H_z, single phase supply with combined pH electrode, range 0-14 pH/0-1400 mv, temperature compensation 0-100°C.

- **Balances:** Manual - single pan, capacity 100-200 g, sensitivity 0.1 mg. Electronic - top loading, precision €0.005 g, range - 0 to 1200 g.
- **Electric Hot Air Oven:** Thermostatically controlled, range 5°C to 250 ± 2°
- **Microscopes**
  - **Dissecting:** two lenses (eyepieces) 10X, 20X.
  - **Laboratory:** with facilities for bright field, dark field, phase contrast, wide angle eyepiece with photomicrographic attachment.
  - **Inverted:** Wide angle eyepieces, quintuple objective turret adjustable specimen 55 mm petridish holder and photomicrographic attachment.
- **Centrifuge** (low speed): Variable electronic speed control, speed indicator, Amp. meter, timer, dynamic break, o starting switch, 230 V 50 H_z.
- **Electric Autoclaves**
  - **Vertical:** with safety valves, pressure gauge, steam-release cock.
  - **Horizontal:** mounted to tubular stand, heavy hinges
- **Steamers:** With immersion heaters of ejection safety device, size: 31 cm x 1 3 cm x 10 cm to 61 cm x 20 cm x 15 cm.
- **Filter Sterilization Equipment:** Syringe filter holder: 2.5 cm
- **Pressure Filter Holder:** 4.7 cm
- **Manestry Stills:** Electrical water stills, 2 lit capacity/hr of 1.5 kw.
- **Double Distilled Water Equipment:** Built-in energy regulator all glass water stills, heat resistant boiling flask with heater.
- **Environmental Growth Cabinets:** Cabinet with controlled temperature, light and humidity, temperature range 4° to 45°C with timer to regulate photoperiods.
- **Gyratory Shakers:** Capacity: 50 to 1000 ml flasks, speed: 80 to 200 rpm.
- **Laminar Air-flow Cabinets:** Constant flow of purified air, sizes: 0.6 m, 1.2 m and 1.8 m.

The other miscellaneous equipments which are required for tissue culture are: air conditioners, arrow heads, bunsen burners, deep-freeze, dissecting needles, glasswares, forceps, florescent lamps/tubes, heaters, hot plates with magnetic stirrer, inoculation cabinets, metal trays and bowls (for transport of cultures), tubes, refrigerators, UV germicidal lamps, wooden or metal racks, etc.

ASEPTIC TECHNIQUE

In *in vitro* condition plant cells and microbes have basically same requirements. When the culture medium contains sugar (as carbon source) it attracts a variety of microorganisms which grow fast than that of the cultured tissue in medium and they ultimately kill the plant cells. It is, therefore, necessary to have complete aseptic condition around the culture equipments which prevents contamination of the culture medium. Following are the three main sources of contamination of the medium and the subsequent methods to

check them:

1. The microorganisms may be present in the nutrient medium at the time of its preparation. These microorganisms can be destroyed by proper plugging and autoclaving the culture tubes/flask. The medium can be completely sterilized by maintaining it at 120° C for about 20 minutes at 15 lb pressure in the autoclave.
2. The explant (plant part to be cultured) may carry microorganisms with it, therefore, the plant part should be surface-sterilized by mercuric chloride (1 to 2%) or by sodium hypochloride solution for 30 minutes.
3. Precautions must be observed to prevent the entry of microorganisms when the plug of a culture is removed during transfer of the plant material to the medium or from one medium to another. The inoculation chamber may be sterilized by UV-radiations.
4. Correct pH of the medium is important. Highly alkaline or acidic pH affects the nutrient uptake in culture tissues. Therefore, the tissue culture medium is adjusted to a pH of 5.6 to 6.0 before autoclaving.
5. Semi-solid and liquid medja are most commonly used for growing plant cells. A high concentration of gelling agent (agar-agar, gelatin, silica gel) makes the medium very hard and decreases the nutrient uptake by the tissues. Agar at 0.8% to 1.0% concentration is widely used.

STERILIZATION OF PLANT TISSUES

It is essential to remove dirt and debris from the plant tissue and should be washed in a weak detergent solution and rinsed several times with distilled water prior to sterilization. Some woody tissues, such as buds and twigs, are cleaned by immersing them briefly in a 70% ethanol solution, which wets and spreads over the tissue surfaces more effectively than a higher concentration alcohol solution.

- **Sodium Hypochlorite (NaOCl):** It is the most common chemical agent used to sterilize plant tissues (0.025%-0.25% NaOCl). Diluted household bleach can also be used for this purpose, which normally contains 5.25% NaOCl. It is equally effective and considerably less expensive.
- **Calcium hypochlorite (CaOCl):** It can be used as a substitute for NaOCl. CaOCl causes slightly less damage to plant tissues but tends to precipitate out of solution. To avoid the accumulation of CaOCl on the plant tissue surfaces, sterilization solutions should be filtered or decanted prior to use.
- **Hydrogen Peroxide (H_2O_2) Solution:** Plant tissues can also be surface sterilized using a H_2O_2 (3%-10%). It is much easier to remove from tissues than NaOCl and CaOCl.
- **Other Substances:** Plant tissue can also be surface-sterilized by **bromine water** (1%-2%), **silver nitrate** ($AgNO_3$ 1%) and **mercuric chloride** ($MgCl_2$ 0.1%-1%).

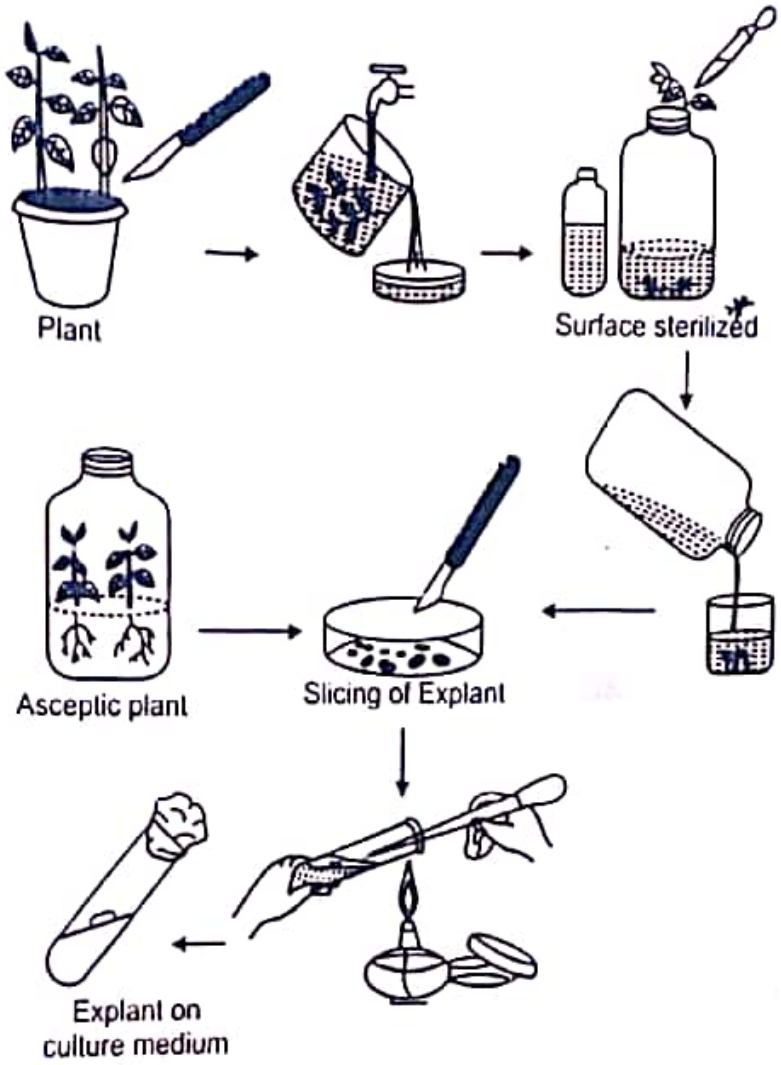


FIG. 3.3: Diagrammatic presentation of the procedure for surface sterilization of plant material and inoculation of explant for culture.

Cleaning (Preparation of Glasswares/ Plastic wares (Autoclavable)

1. Clean the glasswares/plastic wares in 10% commercial detergent liquid or powder for 1 hr and then in HCl for 2 hr.
2. Remove the traces of detergent and acid by thorough washing with tap water
3. Rinse vessels with double distilled water and allow them to dry over night at room temperature.

Sterilization

1. Plug glasswares such as conical flasks or test tubes with non-absorbent cotton or cover by the plastic caps. Wrap the petri dishes with aluminium foil. Place forceps and scalpels in test tubes, plug the tubes with cotton or cover with aluminium foil. Plug the mouth end of the pipettes with cotton.

Wrap them individually in aluminium foil.

2. Autoclave glasswares and instruments at 121°C for 1 hr.
3. For dry-heat sterilization, metal instruments should be sterilized in an oven at 140-160°C for 2 hr.
4. Filter sterilization for heat labile amino acids, vitamins, growth regulators, antibiotics, natural complexes should be through millipore filtration assembly using filter membranes of 0.45 or 0.22 µM porosity. Plug the receiver flask with cotton. Assemble the filtration assembly and wrap the filtration unit with paper or foil. Autoclave the receiver and filtration unit at 121°C for 1 hr. Attach the filtration unit with receiver flask with vacuum pump in a laminar flow bench pour solution to be sterilized into the filtration unit. Apply slight air pressure to start filtration. Transfer the desired volume to sterile flasks under laminar air-flow bench. Use a sterile pipette for drawing filter sterilized solution to autoclaved medium.

Surfactants

- **Tween 20 or Triton X-100:** These are scientific reagent-grade surfactants and are often added in low concentrations (0.05%) to chemical sterilization solutions. Their use ensure that the sterilizing agent come in contact with the entire plant tissue surface.
- **Stirring of the Tissues:** Good surface contact is also facilitated by stirring the tissues during sterilization.
- **Ultrasonic Bath:** It is an effective method to ensure good surface contact during sterilization treatment in an ultrasonic bath like those used to clean dentures.

This technique is particularly useful for sterilizing buds and woody tissues that have many small surface crevices and cracks.

After surface sterilization, a minimum of three sequential rinses with sterile distilled water are recommended to remove any remaining chemical sterilizing agent.

CONTROL OF BACTERIAL AND FUNGAL CONTAMINANTS BY ANTIBIOTICS IN PLANT TISSUE CULTURE

Antibiotics are used to reduce, control, or eliminate contaminants in plant tissue cultures.

It is suggested that antibiotics should not replace careful aseptic technique. Most antibiotics are heat-sensitive and cannot be autoclaved. They are usually dissolved in water or another suitable solvent, filter-sterilized, and added to autoclaved culture media that has cooled to 45°- 50° C. Falkiner (1990) suggested the list of antibiotics which are suitable for plant tissue culture.