ANNAMALAI UNIVERSITY

OPC GPB 001**: Plant Tissue Culture (1+1)**

Practical Manual cum Record

Course teacher

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DEPARTMENT OF GENETICS AND PLANT BREEDING

FACULTY OF AGRICULTURE

ANNAMALAI UNIVERSITY

ANNAMALAI NAGAR-608 002

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OPC GPB 001: Plant Tissue Culture (1+1)

Certificate

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I. D. No. _____ of III B.Sc Agriculture for the course OPC GPB 001- Plant Tissue Culture (1+1) during _____semester, 2019-2020.

External Examiner **Course Teacher**

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Ex. no:1 **Designing a Plant Tissue culture Laboratory** **Page no:**

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Tissue culture is rapidly becoming a commercial method for propagating new cultivars, rare species, and difficult-to-propagate plants. Currently, the demand for micropropagated plants is greater than the supply with some plants. Some growers specialize in only the micropropagation of plantlets; many growers are integrating a tissue culture laboratory into their overall operation. In designing any laboratory, certain elements are essential for a successful operation. The correct design of a laboratory will not only help maintain asepsis, but it will also achieve a high standard of work.

Size and location

Careful planning is an important first step when considering the size and location of a laboratory. It is recommended that visits be made to several other facilities to view their arrangement and operation. A small lab should be set up first until the proper techniques and markets are developed. A convenient location for a small lab is a room or part of the basement of a house, a garage or a remodeled office. The minimum area required for media preparation, transfer and primary growth shelves is about 150 sq ft. Walls may have to be installed to separate different areas.

Larger labs are frequently built as free-standing buildings. Although more expensive to build, the added isolation form adjacent activities will keep the laboratory cleaner.

Consideration should be given to the following:

1. Locate the building away from sources of contamination such as a gravel driveway or parking lot, soil mixing area, shipping dock, pesticide storage, or dust and chemicals from fields.
2. A clear span building allows for a flexible arrangement of walls.
3. The floor should be concrete.
4. Walls and ceiling should be insulated and be covered inside with a water-resistant material.
5. Windows, if desired, may be placed wherever convenient in the media preparation and glassware washing rooms.
6. The heating system should be capable of maintaining a room temperature at 20° C n the coldest part of winter.
7. Accessibility of getting water should be easy
8. Connection to a septic system or sanitary sewer should be provided.
9. Air conditioning for summer cooling may be necessary.
10. Electric service capacity for equipment, lights and future expansion should be calculated. A minimum 100 amp service is recommended.

Glassware washing and storage area

The glassware washing area should be located near the sterilization and media preparation areas. When culture vessels are removed from the growth area, they are often autoclaved to kill contaminants or to soften semi-solid media. The vessels can be easily moved to the washing area if the autoclave or pressure cooker is nearby. Locate the glassware storage area close to the wash area to expedite storage; these areas also need to be accessible to the media preparation area.

The glassware area should be equipped with at least one large sink; two sinks are preferable. Adequate workspace is required on both sides of the sink; this space will be used for glassware soaking tubs and drainage trays. The pipes leading from the sink can be PVC to resist damage from acids and alkalis. Mobile drying racks can be stored nearby and lined with cheesecloth to prevent water dripping and loss of small objects. Locate ovens or hot air cabinets (25 ° C) close to the glassware washing and storage area. Dust-proof cabinets, low enough to allow easy access, can be used in the storage area.

Media preparation and sterilization area

The water source and glassware storage area should be convenient to the media preparation area. Benches, suitable for comfortable working while standing (34 to 36in.) and deep enough (24 in.) to hold equipment listed below are essential. Their tops should be made with molded plastic laminate surfaces that can tolerate frequent cleanings. There is a variety of equipment available for micropropagation laboratories; this equipment is generally located in the media preparation area. The equipment budget will determine the type and amount purchased.

All laboratories need the following basics:

1. **Refrigerator/freezer**-- This is needed to store chemicals and stock solutions. Small laboratories may find it adequate to use countertop refrigerators.
2. **High quality water**--Bottled water can be purchased inexpensively and placed in the media preparation area. Larger businesses may find it economical to obtain distillation or deionization devices; these would normally be located in the glassware washing area. Small, inexpensive, low production Pyrex distillation devices can be purchased by small businesses that want the convenience of a still, but not the cost.
3. **Balances--**High quality balances are essential for a micropropagation laboratory; this is one area where it is difficult to find an inexpensive substitute. A triple beam balance is useful for large amounts over 10 grams, but a balance that can measure down to 2 mg is essential. Most laboratories have both a microbalance and a less sensitive top loading balance; the latter can be used more quickly and efficiently for less sensitive quantities.
4. **Hot plate/stirrer**--At least one hot plate with an automatic stirrer is needed to make semi-solid media. Using a stove and hand stirring the media while it heats can eliminate this purchase; however, the time saved by using a stirring hot plate is worth the money spent.
5. **pH meter**--This is needed to measure media pH. Some laboratories use pH indicator paper, however this method is considerably less accurate and could severely affect the results.
6. **Autoclave**--An autoclave or pressure cooker is a vital part of a micropropagation laboratory. High-pressure heat is needed to sterilize media, water, glassware, and utensils. Certain spores from fungi and bacteria will only be killed at a temperature of 121° C and 15 pounds per square inch (psi). Self-generating steam autoclaves are more dependable and faster to operate.
7. **Optional equipment**--A variety of non-essential equipment is available for tissue culture laboratories; individual needs and equipment cost will determine what can be purchased. Microwave ovens are convenient for defrosting frozen stocks and heating agar media. Dissecting microscopes are useful to have in the laboratory for meristeming, dissecting floral and shoot apices, and observing plant culture growth. Labwashers, or regular dishwashers, can be useful. Automatic media dispensers are helpful when pipetting large volumes of media.

Primary growth room

Temperature is the primary concern in culture rooms; it affects decisions on lights, relative humidity, and shelving. Generally, temperatures are kept 25 ±2 ° C. Heating can be accomplished by traditional heating systems supplemented with heat from light ballasts or space heaters. Cooling the room is usually a greater problem than heating; cooler temperatures can be obtained by installing heat pumps, air conditioners, or exhaust fans. Using outside windows to cool culture rooms invites contamination problems in the summer and humidity problems in the winter.

Some plant cultures can be kept in complete darkness; however, most culture rooms are lighted at 1 Klux (approximately 100ft-c) with some going up to 5 to 10 Klux. The plant species being micropropagated will determine the intensity used. The developmental stage of the plants will also help determine if wide spectrum or cool white fluorescent lights are used. Rooting has been shown to increase with far-red light; therefore, wide spectrum lights should be used during stage III and cool-white lights can be used during Stages I and II. Automatic timers are needed to maintain desired photoperiods. Reflectors can be placed over bulbs to direct their light. Heat generated by the lights may cause condensation and temperature problems.

Relative humidity (RH) is difficult to control inside growing vessels, but fluctuations in the culture room may have a deleterious effect. Cultures can dry out if the room's RH is less than 50%; humidifiers can be used to correct this problem. If the RH becomes too high, a dehumidifier is recommended.

Shelving within primary growth rooms can vary depending upon the situation and the plants grown. Wood is recommended for inexpensive, easy-to-build shelves. The wood for shelves should be exterior particleboard or plywood and should be painted white to reflect the room's light. Expanded metal is more expensive than wood, but provides better air circulation; wire mesh of 1/4 or 1/2 in. hardware cloth can be used but tends to sag under load. Tempered glass is sometimes used for shelves to increase light penetration, but it is more prone to breaking. Air spaces, 2 to 4 in., between the lights and shelves will decrease bottom heat on upper shelves and condensation in culture vessels.

Aseptic transfer area

The aseptic transfer area needs to be as clean as possible. It is preferable to have a separate room for aseptic transfer; this decreases spore circulation and allows personnel to leave shoes outside the room. Special laboratory shoes and coats should be worn in this area. Laminar flow hoods or still-air boxes can be placed in this room and used for all aseptic work. Ultraviolet (UV) lights are sometimes installed in transfer areas to disinfect the room; these lights should only be used when people and plant material are not in the room. Safety switches can be installed to shut off the UV lights when regular room lights are turned on. Surfaces inside the aseptic transfer area should be smooth to minimize the amount of dust that settles. Several electric outlets are to be installed to accommodate balances, flow hoods, microscopes.

Work done

Ex.no: 2 **Instrumentation in biotechnology lab** **Page no:**

Date:

1. Analytical centrifuge: Theodor Svedberg

A centrifuge is equipment that puts an object in rotation around a fixed axis applying a potentially strong force perpendicular to the axis of spin (outward). PRINCIPLE: The centrifuge works using the sedimentation principle, where the centripetal acceleration causes denser substances and particles to move outward in the radial direction. At the same time, objects that are less dense are displaced and move to the center. USES: centrifuge can spin at up to 15,000 rpm to facilitate separation of the different phases of the extraction. In DNA extraction to move precipitated DNA to the bottom of the container and make it stick there, so that the supernatant can be poured off without losing your extract. To separate cell debris from DNA-containing supernatant, so that this supernatant can be removed and DNA can be precipitated out of it.

2. Thermal cycler or PCR

Developed in 1983 by Kary Mullis

Working principle of PCR: A small fragment of the DNA serves as the template for producing the primers that initiate the reaction. One DNA molecule is used to produce two copies, then four, then eight and so forth. There are three major steps in a PCR, which are repeated for 30 or 40 cycles. This is done on an automated cycler, which can heat and cool the tubes with the reaction mixture in a very short time.

3. *Denaturation* at 94°C : During the denaturation, the double strand melts open to single stranded DNA, all enzymatic reactions stop (for example : the extension from a previous cycle).

Annealing at 54°C : The primers are jiggling around, caused by the Brownian motion. Ionic bonds are constantly formed and broken between the single stranded primer and the single stranded template. The more stable bonds last a little bit longer (primers that fit exactly) and on that little piece of double stranded DNA (template and primer), the polymerase can attach and starts copying the template. Once there are a few bases built in, the ionic bond is so strong between the template and the primer, that it does not break anymore.

Extension at 72°C : This is the ideal working temperature for the polymerase. The primers, where there are a few bases built in, already have a stronger ionic attraction to the template than the forces breaking these attractions. Primers that are on positions with no exact match, get loose again (because of the higher temperature) and don't give an extension of the fragment. The bases

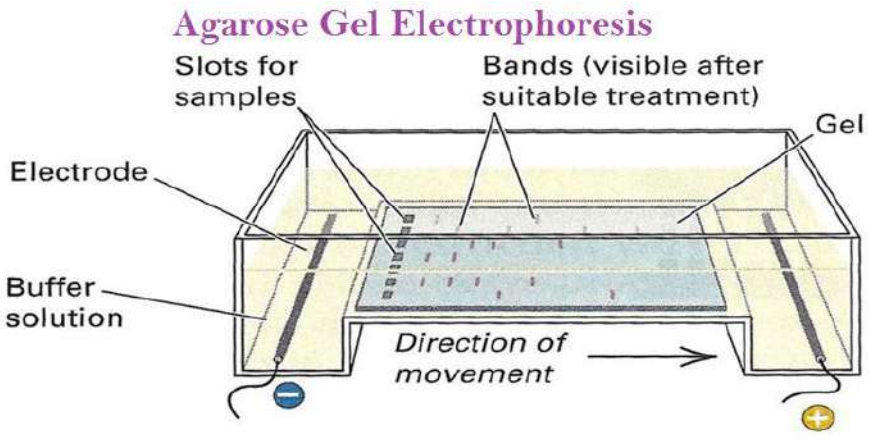
(complementary to the template) are coupled to the primer on the 3' side (the polymerase adds dNTP's from 5' to 3', reading the template from 3' to 5' side, bases are added complementary to the template)

3. Electrophoresis chamber

This is an equipment for separation and analysis of macromolecules (DNA, RNA and proteins) and their fragments, based on their size and charge. Principle:"Electrophoresis" refers to the electromotive force (EMF) that is used to move the molecules through the gel matrix. By placing the molecules in wells in the gel and applying an electric field, the molecules will move through the matrix at different rates, determined largely by their mass. Nucleic acid molecules are separated by applying an electric field to move the negatively charged molecules through a matrix of agarose or other substances. Shorter molecules move faster and migrate farther than longer ones because shorter molecules migrate more easily through the pores of the gel.

Agarose gel: used for seprating DNA fragments of usually 50-20,000 bp in size polyacrylamide gel :Polyacrylamide gels are usually used for proteins and small fragments of DNA (5-500 bp)

Starch :Partially hydrolysed potato starch makes for another non-toxic medium for protein electrophoresis. Invented by: ArneTiselius in the 1931.



4. Micropipetts

Adjustable micropipette that deliver a measured volume of liquid; depending on size, it could be between about 0.1 µl to 1000 µl (1 ml).These pipettes require disposable tips that come in contact with the fluid. PRINCIPLE :These pipettes operate by piston-driven air displacement. A vacuum is generated by the vertical travel of a metal or ceramic piston within an airtight sleeve. As the piston

moves upward, driven by the depression of the plunger, a vacuum is created in the space left vacant by the piston. The liquid around the tip moves into this vacuum (along with the air in the tip) and can then be transported and released as necessary. These pipettes are capable of being very precise and accurate The micropipette was invented and patented in 1960 by Dr.Heinrich Schnitger Marburg, Germany. Afterwards, the co-founder of the biotechnology company Eppendorf, Dr. Heinrich Netheler, inherited the rights and initiated the global and general use of micropipettes in labs.

5. UV transilluminator

To view DNA (or RNA) that has been separated by electrophoresis through an agarose gel. PRINCIPLE : During or immediately after electrophoresis, the agarose gel is stained with a fluorescent dye which binds to nucleic acid. Exposing the stained gel to a UVB light source causes the DNA/dye to fluoresce and become visible.

6. Ultra low temperature freezers

Freezers for -80 to -85°C. There are upright and chest freezers between 300 and 800 L.

Principle: The refrigeration system of the ultra freezers basic cascade refrigeration principle, the choice of two hermetic compressors as high, the compressor of the cryogenic stage.The cryogenic stage system is also equipped with gas heat exchanger, allows low- pressure gas from the evaporator heat exchange with the high-pressure gas condensate evaporator, it will not only reduce the heat load of the condensate evaporator, and the full use of the heat .

Uses: for long term storage for biological samples like DNA, RNA, proteins, cell extracts, or reagents.To reduce the risk of sample damage, these types of samples need extremely low temperatures as -80 to -85°C. Invented & patented by Chuan Weng, Allan Kelly

7. Incubators - An incubator is a device used to grow and maintain microbiological cultures or cell cultures. The incubator maintains optimal temperature, humidity and other conditions such as carbon di oxide and oxygen content of atmosphere inside. Invented by louis Pasteur

PRINCIPLE: an incubator has a compressor that works as a heater as well as cooler and maintains the optimum or required temperature for growth.

8. Vortex mixer is a simple device used commonly in laboratories to mix small vials of liquid. It consists of an electric motor with the drive shaft oriented vertically and attached to a cupped rubber piece mounted slightly off-center. As the motor runs the rubber piece oscillates rapidly in a circular motion.When a test tube or other appropriate container is pressed into the rubber cup (or touched to its edge) the motion is transmitted to the liquid inside and a vortex is created. Principle: vortex, In fluid

dynamics, a vortex is a region in a fluid in which the flow rotates around an axis line, which may be straight or curved.[The vortex mixer was invented by the Kraft brothers (Jack A. Kraft and Harold D. Kraft) while working for Scientific Industries (a laboratory equipment manufacturer).[1] A patent was filed by the Kraft brothers on April 6, 1959 and granted on October 30, 1962.[2] Scientific Industries still makes a version of this original vortex mixer.

9. Pestle and mortar It is used for grinding plant samples which lead to disrupting cellular membranes and specially cell wall. Or in other words to release biological molecules from inside the cell

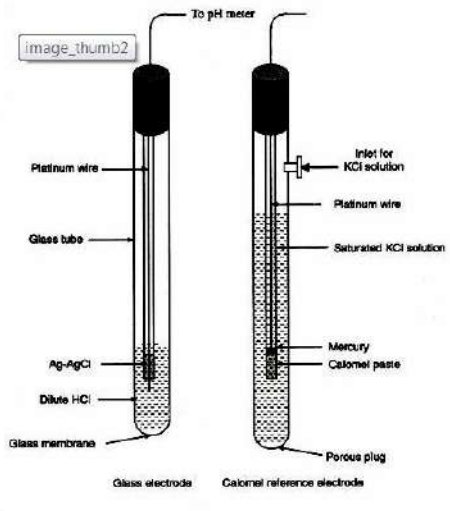
10. Eppendroff tubes Are small capped plastic tubes used for centrifuge or in pcr apparatus. • Available in different volumes like 0.5 ml, 1.5 ml, 2 ml but the most comman size is 1.5 ml

11. gloves and uv glasses or face shields

Laboratory gloves are made of latex and nitrile.They protect the hands of wearer against chemicals which may be corrosive or carcinogenic or hazrdous in any nature. Do not use vinyl gloves which can transmit significant amount of uv. Uv glasses or face shields : use poly carbonate face shields that are rated for uv protection.

12. PH meter:

The pH probe measures pH as the activity of hydrogen ions surrounding a thin walled glass bulb at its tip. The probe produces a small voltage (about 0.06 volt per pH unit) that is measured and displayed as pH units by the meter uses: To adjust pH of different solutions, preparation of buffers and culture media.



13. Autoclave:

Autoclave is used to sterilize medium, glassware and tools for the purpose of plant tissue culture. Sterilization of material is carried out by increasing moist heat (121 °C) due to increased pressure inside the vessel (15-22 psi, pounds per square inch or 1.02 to 1.5 kg/cm^2) for 15 minutes for routine sterilization. Moist heat kills the microorganism and makes the material free from microbes.

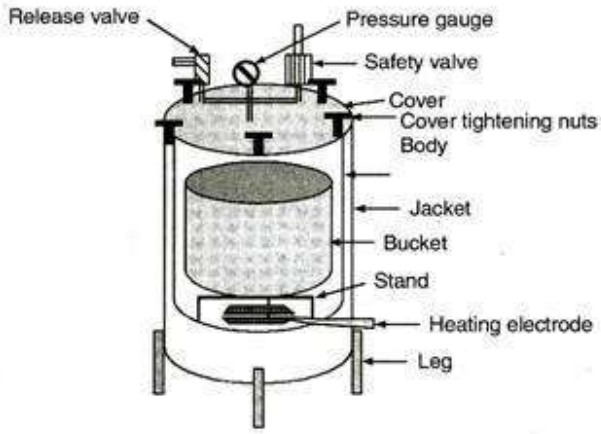


Fig. 25.2. A schematic diagram of laboratory autoclave.

15. Laminar Air Flow Bench:

Table for aseptic manipulations in tissue culture. It has High Efficiency Particulate Air (HEPA) Filters, which allow air to pass but retain all the particles and micro-organisms (small pore size (0.3 μm) with 99.97-99.99% efficiency). Air blown from inside to outside. Equipment is fitted with UV light and visible light source. UV is switched-on for 30 minutes before starting the work to make area free from microbes.

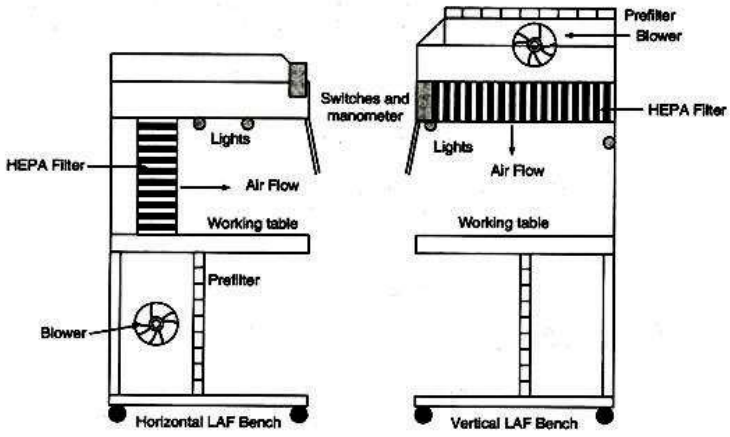


Fig. 25.3. Line diagram of horizontal laminar air flow bench.

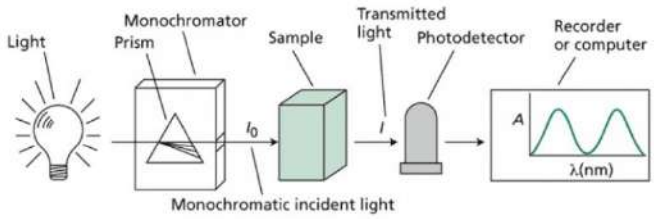
16.Light Microscopy:

This is required for routine observations of cells, cellular differentiation and pigmentation. Fluorescence microscopy for the selection of fluorescing secondary metabolite rich cells. A good

microscope has not only good magnifying power but also good resolving power. The resolving power of a microscope depends upon the wavelength of light and numerical aperture (N.A.)

17. Spectrophotometer: used for quantitative analysis in various areas.
Spectrometer: transmits a straight beam of light (photons) through a monochromator (prism) Then a wavelength selector (slit) transmits only the desired wavelengths (depending on compound)
Photometer: light passes through the solution of a sample , the photometer detects the amount of photons that is absorbed and then sends a signal to a digital display.

UV-visible spectrophotometer: uses light over the ultraviolet range (185 - 400 nm) and visible range (400 - 700 nm) of electromagnetic radiation spectrum.
IR spectrophotometer: uses light over the infrared range (700 - 15000 nm) of electromagnetic radiation spectrum.



11. LUX Meter
Light intensity can be measured by photometer or lux meter. A photometer consists of photoelectric cell and a micro-ammeter. Photoelectric cell is sensitive for light and converts light into current. Micro-ammeter shows reading due to this current and its needle moves.
10. Hygrometer:
The RH present in the culture room is measured by hair hygrometer. As the name suggests a chemically treated hair elongates with increased humidity and shortens with dryness (similar to mercury in thermometer). It is calibrated from 0 to 100% RH. At lower humidity, medium dries rapidly whereas at higher humidity chances of fungal growth over all surfaces and cotton plugs is increased. Therefore, about 60% RH is maintained in the culture room

Ex.no: 3 **Sterile (Aseptic) Techniques** **Page no:**

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Aseptic technique is absolutely necessary for the successful establishment and maintenance of plant cell, tissue and organ cultures. The in vitro environment in which the plant material is grown is also ideal for the proliferation of microorganisms. In most cases the microorganisms outgrow the plant tissues, resulting in their death. Contamination can also spread from culture to culture. The purpose of aseptic technique is minimizing the possibility that microorganisms remain in or enter the cultures.

The environmental control of air is also of concern because room air may be highly contaminated. Example: Sneezing produces 100,000 - 200,000 aerosol droplets which can then attach to dust particles. These contaminated particles may be present in the air for weeks. Air may also contain bacterial and fungal spores, as do we.

Sterilization and Use of Supplies and Equipment:

A. Sterilizing tools, media, vessels etc.

1. Autoclaving

Autoclaving is the method most often used for sterilizing heat-resistant items and our usual method for sterilizing items. In order to be sterilized, the item must be held at 121°C, 15 psi, for at least 15 minutes. It is important that items reach this temperature before timing begins. Therefore time in the autoclave will vary, depending on volume in individual vessels and number of vessels in the autoclave. Most autoclaves automatically adjust time when temperature and psi are set, and include time in the cycle for a slow decrease in pressure. There are tape indicators that can be affixed to vessels, but they may not reflect the temperature of liquid within them. There are also “test kits” of microorganisms that can be run through the autoclave cycle and then cultured.

Empty vessels, beakers, graduated cylinders, etc., should be closed with a cap or aluminum foil. Tools should also be wrapped in foil or paper or put in a covered sterilization tray. It is critical that the steam penetrate the items in order for sterilization to be successful.

2. Autoclaving and Filter-sterilizing Media and Other Liquids

Two methods (autoclaving and membrane filtration under positive pressure) are commonly used to sterilize culture media. Culture media, distilled water, and other heat stable mixtures can be autoclaved in glass containers that are sealed with cotton plugs, aluminum foil, or plastic closures. However, solutions that contain heat-labile components must be filter-sterilized. For small volumes of liquids (100 ml or less), the time required for autoclaving is 15-20 min, but for larger quantities (2-4 liter), 30-40 min is required to complete the cycle. The pressure should not exceed 20 psi, as higher pressures may lead to the decomposition of carbohydrates and other components of a medium. Too high temperatures or too long cycles can also result in changes in properties of the medium.

Organic compounds such as some growth regulators, amino acids, and vitamins may be degraded during autoclaving. These compounds require filter sterilization through a 0.22 μm membrane. Several manufacturers make nitrocellulose membranes that can be sterilized by autoclaving. They are placed between sections of a filter unit and sterilized as one piece. Other filters (the kind we use) come pre-sterilized. Larger ones can be set over a sterile flask and a vacuum is applied to pull the compound

dissolved in liquid through the membrane and into the sterile flask. Smaller membranes fit on the end of a sterile syringe and liquid is pushed through by depressing the top of the syringe. The size of the filter selected depends on the volume of the solution to be sterilized and the components of the solution.

Nutrient media that contain thermo labile components are typically prepared in several steps. A solution of the heat-stable components is sterilized in the usual way by autoclaving and then cooled to 35°-50° C under sterile conditions. Solutions of the thermo labile components are filter-sterilized. The sterilized solutions are then combined under aseptic conditions to give the complete medium.

In spite of possible degradation, however, some compounds that are thought to be heat labile are generally autoclaved if results are found to be reliable and reproducible. These compounds include ABA, IAA, IBA, kinetin, pyridoxine, 2-ip and thiamine are usually autoclaved.

3. Ethylene Oxide Gas

Plastic containers that cannot be heated are sterilized commercially by ethylene oxide gas. These items are sold already sterile and cannot be resterilized. Examples of such items are plastic petri dishes, plastic centrifuge tubes etc.

4. UV Radiation

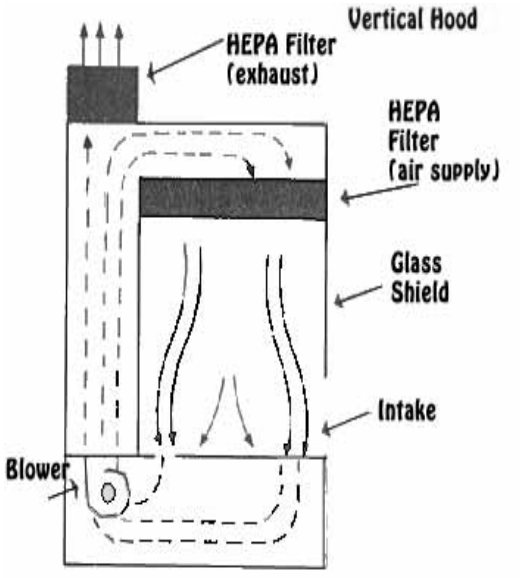
It is possible to use germicidal lamps to sterilize items in the transfer hood when no one is working there. We do not do this. UV lamps should not be used when people are present because the light is damaging to eyes and skin. Plants left under UV lamps will die.

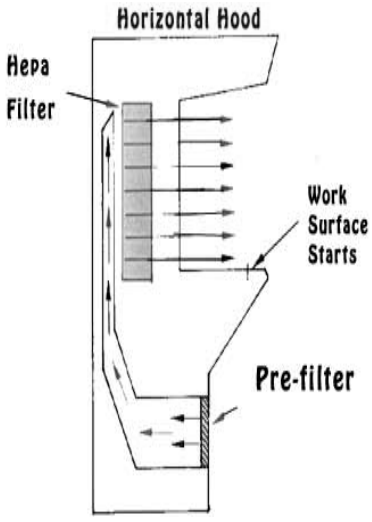
5. Microwave

It is also possible to sterilize items in the microwave; we do not do this.

6. The Transfer 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 Energy Particle Air) filter with a uniform velocity of 90 ft/min across the work surface. The air is filtered by a **HEPA (high efficiency particulate air) 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.





Working in the Transfer Hood:

- The hood should remain on continuously. If for some reason it has been turned off, turn it on and let it run for at least 15 minutes before using.
- Make sure that everything needed for the work is in the hood and all unnecessary things are removed. As few things as possible should be stored in the hood.
- Check the bottom of the hood to make sure there is no paper or other debris blocking air intake.
- Remove watches, etc., roll up long sleeves, and wash hands thoroughly with soap (preferably bactericidal) and water.
- Spray or wipe the inside of the transfer hood (bottom and sides, not directly on the filters) with 70% EtOH. Others use disinfectants such as Lysol®. Wipe the work area and let the spray dry.
- Wipe hands and lower arms with 70% EtOH. It is not necessary to flame them (This is a joke.).
- Spray everything going into the sterile area with 70% ethanol. For example, spray bags of petri dishes with 70 % alcohol before you open them and place the desired number of unopened dishes in the sterile area.
- Work well back in the transfer hood (behind the line). Especially keep all flasks as far back to the back of the hood as possible. Movements in the hood should be contained to small areas. A line drawn across the distance behind which one should work is a useful reminder.
- Make sure that materials in use are to the side of your work area, so that airflow from the hood is not blocked.
- Don't touch any surface that is supposed to remain sterile with your hands. Use forceps, etc.
- Instruments (scalpels, forceps) can be sterilized by flaming - dipping them in 95% Ethanol and then immediately placing them in the flame of an alcohol lamp or gas burner. This can be dangerous if the vessel holding the alcohol tips over and an alcohol fire results. A fairly deep container, like a coplin-staining jar, should be used to hold the ethanol. Use enough ethanol to submerge the business ends of the instruments but not so much that you burn your hands. Some people wear gloves in the hood for certain procedures. If you do this, be very careful not to get them near the flame. Other methods of sterilization that do not require alcohol are with a bacticinerator or glass bead sterilizer. There is not as much risk from fire with these, but the instruments can still get extremely hot, causing burns.
- Arrange tools and other items in the hood so that your hands do not have to cross over each other while working. For a right-handed person, it is best that the flame, alcohol for flaming, and tools be placed on the right. The plant material should be placed to the left. All other items in the hood should be arranged so that your work area is directly in front of you, and between 8 and 10 inches in from the front edge. No materials should be placed between the actual work area and the filter. Keep as little in the hood as possible.
- Plant material should be placed on a sterile surface when manipulating it in the hood. Sterile petri dishes (expensive), sterile paper towels, or sterile paper plates work fine. Pre-sterilized plastic dishes have two sterile surfaces-the inside top and inside bottom.

- Sterilize your instruments often, especially in between individual petri plates, flasks, etc. The tools should be placed on a holder in the hood to cool or should be cooled by dipping in sterile water or medium before handling plant tissues.
- Wipe up any spills quickly; use 70% EtOH for cleaning. Clean hood surface periodically while working.
- Use of glass or plastic pipettes: Glass pipettes are put into containers or wrapped and then autoclaved. Plastic pipettes are purchased presterilized in individual wrappers. To use a pipette, remove it from its wrapper or container by the end opposite the tip. Do not touch the lower two-thirds of the pipette. Do not allow the pipette to touch any laboratory surface. Insert only the untouched lower portion of the pipette into a sterile container.
- Sterilize culture tubes with lids or caps on. When you open a sterile tube, touch only the outside of the cap, and do not set the cap on any laboratory surface. Instead, hold the cap with one or two fingers while you complete the operation, and then replace it on the tube. This technique usually requires some practice, especially if you are simultaneously opening tubes and operating a sterile pipette. After you remove the cap from the test tube, pass the mouth of the tube through a flame. If possible, hold the open tube at an angle. Put only sterile objects into the tube. Complete the operation as quickly as you reasonably can, and then flame the mouth of the tube again. Replace the lid.
- Inoculating loops and needles are the primary tools for transferring microbial cultures. We use plastic ones that come sterile. If you are moving organisms from an agar plate, touch an isolated colony with the transfer loop. Replace the plate lid. Open and flame the culture tube, and inoculate the medium in it by stirring the end of the transfer tool in the medium. If you are removing cells from a liquid culture, insert the loop into the culture. Even if you cannot see any liquid in the loop, there will be enough cells there to inoculate a plate or a new liquid culture.

- If you don't have to be careful about the volume you transfer, a pure culture or sterile solution can be transferred to a sterile container or new sterile medium by pouring. For example, we do not measure a specific volume of medium when we pour culture plates, although after you have done it for a while, you become pretty consistent. Remove the cap or lid from the solution to be transferred. Thoroughly flame the mouth of the container, holding it at an angle as you do so. Remove the lid from the target container. Hold the container at an angle. Quickly and neatly pour the contents from the first container into the second. Replace the lid.
- If you must transfer an exact volume of liquid, use a sterile pipette or a sterile graduated cylinder. When using a sterile graduated cylinder, complete the transfer as quickly as you reasonably can to minimize the time the sterile liquid is exposed to the air.

- Remove items from the hood as soon as they are no longer needed. All cultures must be sealed before leaving the hood.
- When transferring plant cultures, do contaminated cultures last. Situate the cultures so that the contaminated part is closest to the front of the hood.
- Place waste in the proper containers: Empty (e.g. after transfer) or old petri plates used in transformation experiments go in the big bag to be autoclaved, as do other disposable that were in contact with recombinant bacterial or plant material. All needles go in the sharps box, needles used with bacteria get autoclaved. Small bags used in the hood for waste go in the big bag to be

autoclaved; do not overfill the small bags or leave full bags in or on the hood for someone else to dispose of. Glassware that comes in contact with bacteria is placed in a separate pan to be autoclaved.

- When finished in the hood, clean up after yourself. Remove all unnecessary materials and wipe the hood down with 70% EtOH.
- Be sure when you are finished that you turn off the gas to the burner!
- It is pointless to practice good sterile technique in a dirty lab. Special problems are contaminated cultures, dirty dishes and solutions where microorganisms can grow.
- Check cultures every 3-5 days for contamination.

B. Surface-sterilizing of Plant Material

1. Preparation of Stock Plants

Prior good care of stock plants may lessen the amount of contamination that is present on explants. Plants grown in the field are typically more "dirty" than those grown in a greenhouse or growth chamber, particularly in humid areas like Florida. Overhead watering increases contamination of initial explants. Likewise, splashing soil on the plant during watering will increase initial contamination. Treatment of stock plants with fungicides and/or bacteriocides is sometimes helpful. It is sometimes possible to harvest shoots and force buds from them in clean conditions. The forced shoots may then be free of contaminants when surface-sterilized in a normal manner. Seeds may be sterilized and germinated in vitro to provide clean material. Covering growing shoots for several days or weeks prior to harvesting tissue for culture may supply cleaner material. Explants or material from which material will be cut can be washed in soapy water and then placed under running water for 1 to 2 hours.

2. Ethanol (or Isopropyl Alcohol)

Ethanol is a powerful sterilizing agent but also extremely phytotoxic. Therefore, plant material is typically exposed to it for only seconds or minutes. The more tender the tissue, the more it will be damaged by alcohol. Tissues such as dormant buds, seeds, or unopened flower buds can be treated for longer periods of time since the tissue that will be explanted or that will develop is actually within the structure that is being surface-sterilized. Generally 70% ethanol is used prior to treatment with other compounds.

3. Sodium Hypochlorite

Sodium hypochlorite, usually purchased as laundry bleach, is the most frequent choice for surface sterilization. It is readily available and can be diluted to proper concentrations. Commercial laundry bleach is 5.25% sodium hypochlorite. It is usually diluted to 10% - 20% of the original concentration, resulting in a final concentration of 0.5 - 1.0% sodium hypchlorite. Plant material is usually immersed in this solution for 10 - 20 minutes. A balance between concentration and time must be determined empirically for each type of explant, because of phytotoxicity.

4. Calcium Hypochlorite

Calcium hypochlorite is used more in Europe than in the U.S. It is obtained as a powder and must be dissolved in water. The concentration that is generally used is 3.25 %. The solution must be filtered prior to use since not the entire compound goes into solution. Calcium hypochlorite may be less injurious to plant tissues than sodium hypochlorite.

5. Mercuric Chloride

Mercuric chloride is used only as a last resort in the U.S. It is extremely toxic to both plants and humans and must be disposed of with care. Since mercury is so phytotoxic, it is critical that many rinses be used to remove all traces of the mineral from the plant material.

6. Hydrogen Peroxide

The concentration of hydrogen peroxide used for surface sterilization of plant material is 30%, ten times stronger than that obtained in a pharmacy. Some researchers have found that hydrogen peroxide is useful for surface-sterilizing material while in the field.

7. Enhancing Effectiveness of Sterilization Procedure

- Surfactant (e.g.Tween 20) is frequently added to the sodium hypochlorite.
- A mild vacuum may be used during the procedure.
- The solutions that the explants are in are often shaken or continuously stirred.

8. Rinsing

After plant material is sterilized with one of the above compounds, it must be rinsed thoroughly with sterile water. Typically three to four separate rinses are done.

9. Use of Antibiotics and Fungicides in Vitro

We have found that the use of antibiotics and fungicides in vitro is not very effective in eliminating microorganisms and these compounds are often quite phytotoxic.

Work done

Ex.no: 4 **Preparation of MS medium** **Page no:**

Date:

The simplest method of preparing media is the use of commercially available dry powdered media, containing inorganic salts, vitamins and amino acids. The powder is dissolved in purified water (10% less than the final volume of the medium), and after adding sugar, agar and other desired supplements, the final volume is made up with purified water. The pH is adjusted, and the medium autoclaved.

1. Preparation of stock solutions

For Murashige and Skoog (MS) medium, stock solutions of macronutrients, minor nutrients, micronutrients, iron, KI and vitamins are prepared. The concentration of the stock solutions depends of the need and utility. The stock solutions are generally stored at 4° C and should be checked by visual inspection before earth use. It is preferable to prepare the stock solutions once in every two months. The stock solutions should be labeled with following details.

1. Name of the compound
2. Formula of the compound
3. Concentration of the compound
4. Date of preparation
5. Initials of the person who prepares it

A. Macronutrient stock:

The salts of nitrogen, potassium, phosphorus, magnesium and sulphur are added one by one to the double distilled (DD) water and finally the volume is made up. The calcium salt is not added to the above as it causes precipitation. Hence stock of calcium is prepared separately.

B. Minornutrient stock:

The salts of manganese, zinc and boric acid are weighed and dissolved in DD water constitute micronutrient stock. The micronutrient stock is prepared at higher concentration *viz* 100x, 200x or 400x as the quantity of chemical required is minimum. Preparation of stock of higher concentrations will lead to precision in weighing the chemicals.

C. Micronutrient stock

Micronutrient stock is also prepared at higher concentration of 400x as the salts of sodium molybdate, copper sulphate and cobalt chloride are to be weighed in milligrams.

D. Iron stock

Availability of iron during the *in vitro* growth is essential. Iron when chelated with EDTA, it remains available upto a pH of 7.6-8.0. Iron EDTA stock has to be prepared in the following manner:

Weigh required quantity of $FeSO_4.7H_2O$ and Na_2 EDTA and dissolve in DD water in different containers and warm both the solutions separately. Hot solution of EDTA should be added to hot

solution of Fe_2SO_4 and make up the final volume. The chelating reaction will be completed on autoclaving. This stock solution should be stored in amber colored bottles to protect it from photo oxidation.

E. KI stock

KI is weighed and dissolved separately. It is stored in amber colored bottled due to its photosensitivity.

F. Vitamins stock

Plants growth under *in vitro* conditions requires vitamins like thiamine, nicotinic acid and pyridoxine. The vitamins are weighed one by one and dissolved in DD water.

G. Stock solution of growth regulators

Auxins

Auxins are generally soluble in either or IN NAOH. Auxin like IAA, IBA, 2,4-D are dissolved in the solvent ethanol and diluted using DD water. For easy dissolving, auxins dissolved in the ethanol are added to the water containing few drops of NaOH, drop by drop. The solvent for NAA is IN NAOH and the diluent is DD water.Cytokinins

Cytokinins are generally dissolved in dilute HCl or NAOH, (1 or 0.5 N HCl or IN NAOH). The solvent for BAP and Kinetin is NaOH and this is diluted in DD water.

Gibberellic acid

GA should be dissolved in water and pH adjusted to 5.7. GA is heat labile and must be fitter sterilized. Compared to auxins and cytokinins, gibberellins are rarely used.

Volume of stock required = Required concentration X Media volume

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Concentration of stock solution

Stock solutions of MS Medium

Ingredients	Final composition in the medium (mg/l)	Stock solution (W/V)	Volume of the stock to be taken per litre of medium
Macro Nutrients (10X) NH_4NO_3 KNO_3 $CaCl_2$ $2H_2O$ $MgSO_4$. $7H_2O$ KH_2PO_4	 1650 1900 440 370 170	 16.50 g 19.00 g 4.40 g 3.70 g 1.70 g in 250 ml	25 ml
Minor Nutrients (100X) MnSO4 4H2O ZnSO4 4H2O H3BO3	 22.3 8.6 6.2	 2.23 g 0.86 g 0.62 g in 250 ml	2.5 ml
Micro Nutrients (100X) Na2MoO4. 2H2O CuSO4 5 H2O CoCl2 6 H2O	 0.250 0.025 0.025	 25.0 mg 2.5 mg 2.5 mg in 100 ml	1.0 ml
KI (100X) KI	 0.83	 0.083 g in 250 ml	2.5 ml
Iron Stock (50X) Na_2EDTA $FeSO_4$ $7H_2O$	 37.25 27.85	 1.863 g 1.393 g in 250 ml	5.0 ml
MS Vitamins (100X) Nicotinic Acid Pyridoxine.HCl Thiamine. HCl Glycine	 0.5 0.5 0.1 2.0	 50 mg 50 mg 10 mg 20 mg in 100 ml	1.0 ml
Carbon source-source	-	-	30g/l
Solidifying agent-agar	-	-	15g/l

2. Steps involved Preparation of MS medium for 1000 ml

Procedure:

1. Take double distilled water in a 1000 ml bottle (400 ml-500ml of DD water for 1 litre media)
2. To this, add required quantity of stock solutions of Macro, micro, iron, vitamins and aminio acids to obtain the specified concentration of each nutrient.
3. Add the required volume of growth regulator stock solutions to the medium
4. Dissolve required quantity of carbon source like sucrose @ 30g/l as per medium composition.
5. Adjust the pH of the medium between 5.5 to 5.8 by using 0.1N NaOH or KOH or 0.5 N HCl
6. Make up the volume using DD H2O to 1000 ml
7. For solid media preparation, weigh the gelling agents like agar (15 g/l), or phytagel (4 g/l)
8. Melt in a microwave oven or stove and distribute to containers.
9. Autoclave the media at 121 C at 20 psi for 20 min. sufficient air volume must be provided to prevent the media from boiling over.
10. Pour the media to the Petri plates/test tubes when it is at bearable warmth.

Note:

Growth regulator stock solutions, vitamins and amino acids may be filter sterilized and added to the autoclaved media when it is at bearable warmth. When filter sterilized components are added to the medium, the volume of autoclaved medium and filter sterilized compound should sum up to the final desired volume and finally it is dispensed into labeled sterile containers under aseptic conditions.

Work done

Ex.no: 5 **Plant Growth Regulators** **Page no:**

Date:

A plant hormone can be defined as a small organic molecule that elicits a physiological response at very low concentrations (e.g., <1mM and often <1uM). As in animals, the definition classically includes synthesis at one site and transport to and action at a different site, but also as in animals, plant hormones are not necessarily translocated. For example, ethylene may bring about changes in the same tissue or even the same cell in which it is synthesized. Plant hormones are low-molecular-weight chemicals that only provide an on/off signal, although they do stimulate a cascade of events in the cell leading to a response. Therefore, although the term plant hormone or phytohormone has been used for many years, it is an inaccurate term and it is better to refer to these substances as plant growth regulators (PGRs) (some say planth growth substances), which have the following characteristics:

- Synthesized by plants and broadly distributed within the plant kingdom.
- Show specific biological activity at very low concentrations.
- Display multiple functions in plants.
- Play a fundamental role in regulating physiological phenomena in vivo in a dose-dependent manner, which may change due to changes in the sensitivity of the plant tissue during development or due to environment.
- Unlike mammalian hormones, different PGRs may interact, either synergistically or antagonistically, to produce a particular effect.

The five classical hormones:

- Auxin
- Cytokinin
- Gibberelin
- Abscisic acid
- Ethylene

These are all small molecules ranging from 28 Da (ethylene) to 346 Da (GA), all synthesized by the plant, and all active at 10^{-6} to 10^{-8} M. They are distributed within tissues from cell to cell, as in the case of auxin, *via* vascular bundles (as in the case of cytokinin), or via the intercellular space (ethylene).

Additional substances gaining recognition as PGRs:

- Polyamines
- Jasmonates
- Salicylic acid
- Brassinosteroids

- others

List of plant growth regulators and their functions

Hormone	Categories	Function in Plant Tissue Culture
Auxins	**Indole-3-Acetic Acid Indole-3-Butyric Acid Indole-3-Butyric Acid, Potassium Salt α-Naphthaleneacetic Acid 2,4-Dichlorophenoxyacetic Acid p-Chlorophenoxyacetic acid Picloram Dicamba**	**Adventitous root formation (high concen) Adventitious shoot formation (low concen) Induction of somatic embryos Cell Division Callus formation and growth Inhibition of axillary buds Inhibition of root elongation**
Cytokinins	**6-Benzylaminopurine 6-Y,Y-Dimethylallylaminopurine (2iP) Kinetin Thidiazuron (TDZ) N-(2-chloro-4-pyridyl)-N'Phenylurea Zeatin Zeatin Riboside**	**Adventitious shoot formation Inhibition of root formation Promotes cell division Modulates callus initiation and growth Stimulation of axillary's bud breaking and growth Inhibition of shoot elongation Inhibition of leaf senescence**
Gibberellins	**Gibberellic Acid**	**Stimulates shoot elongation Release seeds, embryos, and apical buds from dormancy Inhibits adventitious root formation Paclobutrazol and ancymidol inhibit gibberellin synthesis thus resulting in shorter shoots, and**
Abscisic Acid	**Abscisic Acid**	**Stimulates bulb and tuber formation Stimulates the maturation of embryos Promotes the start of dormancy**
Polyamines	**Putrescine Spermidine**	**Promotes adventitious root formation Promotes somatic embryogenesis Promotes shoot formation**

<u>Preparation of PGR stock solutions</u>

Procedure: To prepare a 1 mg/ml stock solution:

Weigh 100 mg of the plant growth regulator to a 100 ml volumetric flask

Add 2-5 ml of solvent to dissolve the powder completely

Once completely dissolved, bring the volume to 100 ml with double processed water.

Store the stock solution as recommended in freezer.

Note: Stirring the solution while adding water may be required to keep the material in solution.

Solvent for dissolution
Auxin like IAA, IBA, 2,4-D are dissolved in the solvent ethanol or 1N NAOH
Cytokinins are dissolved in dilute 0.5 N HCl or IN NAOH

Formula

$$V_1N_1 = V_2N_2$$

Therefore

$$V_1 = \frac{V_2N_2}{N_1}$$

V1 = Volume to be pipetted from stock
N1 = Concentration of stock prepared
V2 = volume of media prepared
N2 = Required stock concentration

$$\text{Volume of stock solution Required} = \frac{\text{Desired hormone concentration} \times \text{Volume of media required}}{\text{Stock solution concentration}}$$

STOCK SOLUTION DILUTION CHART

To use this chart:

1. Determine the final concentration of the hormone/vitamin etc. desired in the culture medium. In column A, locate the final concentration desired under the heading corresponding to the quantity of medium you will prepare.
2. Once you have located the desired final concentration the go across the chart to column B to determine the concentration of stock solution to prepare.

3. Find the volume of stock solution to use to achieve the final desired concentration in the medium in column C.

PGR Chart

B Concentration Of Stock Solution	C Amount to use (ml)	A Concentration Of Final Solution (Mg/L)				
		250 ml	500 ml	1 litre	2 litre	10 litre
0.01 mg/mL	0.1	0.004	0.002	0.001	0.0005	0.0001
	0.5	0.02	0.01	0.005	0.025	0.005
	1.0	0.04	0.02	0.01	0.005	0.001
	10.0	0.4	0.2	0.1	0.05	0.01
0.1 mg/mL	0.1	0.04	0.02	0.01	0.005	0.001
	0.5	0.2	0.1	0.05	0.25	0.05
	1.0	0.4	0.2	0.1	0.05	0.01
	10.0	4.0	2.0	1.0	0.5	0.1
1.0 mg/mL	0.1	0.4	0.2	0.1	0.05	0.01
	0.5	2.0	1.0	0.5	0.25	0.5
	1.0	4.0	2.0	1.0	0.5	0.1
	10.0	40.0	20.0	10.0	5.0	1.0
10.0 mg/mL	0.1	4.0	2.0	1.0	0.5	0.1
	0.5	20.0	10.0	5.0	2.5	0.5
	1.0	40.0	20.0	10.0	5.0	1.0
	10.0	400.0	200.0	100.0	50.0	10.0

Work done

1. Units and formulae
2. Calculations

Ex.no: 6 **Meristem Tip Culture** **Page no:**
Date:

Meristem tip culture is used successfully to remove viruses, bacteria, and fungi from plants. In a majority of cases heat therapy is combined with meristem tip culture in order to produce the greatest number of plants that are "virus free".

A few words of caution are required, particularly in regard to the terminology. The term *meristem*, *shoot tip*, *meristem tip* are often interchanged. Here we will use the term *shoot tip* to refer to an apical tip ranging from 1-3 cm. The *meristem* is strictly the meristematic dome without any primordial leaves. The term *meristem tip* will be used to denote the meristem together with 1-2 primordial leaves and measuring between 0.1 and 0.5 cm in height. The term *virus-free* is a misnomer. After successful heat therapy and meristem tip culture

One can only say that a plant is free from the actual viruses for which one tested. *Pathogen-free* is another term that is misleading. To be more exact one should use the term "specific virus-free" or 'specific pathogen-free'. Plants are kept in special growth chambers at 36^{o}C -38 ^{o}C for 4-6 weeks. One must be careful with this treatment because it can be injurious to the plants, especially if they are already weakened by virus infection.

Why virus eradication works

Several hypotheses exist to explain why heat therapy and meristem tip culture when used together are effective in eradicating viruses.

1. Virus distribution is uneven in a plant and is much less in a meristem.
2. Viruses cannot travel quickly enough through plasmodesmata to keep up with actively growing tip.

Size of explant

Only the meristematic dome and 1 pair of subtending leaves should be excised. If larger pieces are taken, it is likely that the virus will be transmitted. The size of a meristem plus the subtending leaves ranges from 0.1-0.5 mm. The apical dome itself measures from 0.1-0.25 mm depending on the species. There is a balance in size. The meristem tip must be small enough to eradicate viruses and other pathogens, yet large enough to develop into a shoot. Although roots may form on the shoot directly in the same medium, often the shoot has to be transferred to another medium in order for roots to develop. Heat therapy combined with meristem tip culture is able to eradicate viruses, bacteria, and fungi but does not remove viroids. Unlike viruses, viroids are RNA

without a protein coat – thus they are known as 'naked' RNA and are very difficult to eradicate. Usually the infected plant must be destroyed.

Plants in which virus eradication is commonly used

Garlic, pineapple, cymbidium orchid, carnation, hyacinth, sweet potato, lily, apple, banana, narcissus, gooseberry, raspberry, potato, grape, dahlia, strawberry, cassava, perlagonium (geranium) sugarcane. Ginger.

Protocol

Developmental Stages of Plantlets

Stage I - establishment of explant (meristematic dome or shoot tips): can be cultured in ms basal medium supplemeted with cytokinin for-shoots initiate.

Stage II – multiplication and elongation of shoots: Rapid multiplication of the shoots can be attained in regeneration medium containing MS + BAP or kinetin. The shoots can be elongated on MS Medium containing GA_3

Stage III – rooting initiation: Individual shoots can be separated and rooted on rooting medium containing MS medium supplemented with IBA or IAA.

Stage IV – acclimatization: The rooted shoots should be transferred to the pots (10 cm dia) containing a mixture of FYM: sand mixed in 1:1 ratio. Initially the plantlets should be covered with polythene covers to maintain high relative humidity. After 15 - 20 days, when they show initial signs of establishment, the covers can be temporarily removed daily for few hours and the plants should be sprayed with H_2O. After four weeks, the covers can be removed completely and the plants can be kept in glass - house and gradually exposed to normal conditions.

Work done

Ex:no 7 **Micropagation of Banana** **Page No:**

Date:

Banana is a globally important fruit crop with 97.5 million tones of annual production. In India, banana contributes 37% of the total fruit production and ranks second in importance next to mango. Edible bananas do not produce seeds and are traditionally grown vegetatively though suckers (5-10 depending on the variety). However conventional plant materials are not the ideal propagule, because they carry weevils, fungal pathogens, nematodes and viruses. The other disadvantages includes low rate of multiplication, bulkiness and poor phytosanitary quality.

In Vitro propagation of bananas provides following advantages over traditional propagation.

1. Rapid multiplication of desirable plants (one meristem can yield thousands of clones in months)
2. Uniform size and age of plants
3. Availability of disease –free (free of fungal, bacterial, viral and parasitic infections) material
4. Available throughout the year.
5. Short harvest interval due to faster growth in early growing stages.
6. High quality fruit bunches.
7. Rapid dissemination of new plant material all the year round throughout the world.
8. Shipment of micropropagated bananas is easier as it occupies less space.
9. Micropropagation is much useful in varieties which are slow multipliers and shy suckering types

The process of Micropropagation in banana involves following steps:

1.Explant preparation (shoot tip/rhizome bud)

2. Culture initiation and multiple shoot induction

3. Subculturing

4. Rooting of the shoots

5. Hardening

Explant preparation

Suckers are preferred as explants, as they are in an actively growing phase and can be taken without destroying the mother plant. Healthy suckers should be selected from true-to-type mother plants, which are devoid of bacterial and viral diseases like fusarial wilt, banana bract mosaic poty virus and banana streak badnavirus. The plants are indexed for the viruses using ELISA test.

Small and healthy suckers are collected from the banana plants indexed for diseases. The soil adhering to the rhizomes are removed by repeated washing with water. The rhizomes are removed by repeated washing with water. The rhizome bud or shoot tip is obtained by repeated trimming off of the corm by unwhorling of leaf sheath and a small portion of rhizome. The tissues are trimmed until shoot tip of 4cm long with rhizome length of 3cm and a width of 2.5cms is obtained. These shoot tips are stored immediately in antioxidant solution (100mg ascorbic acid+150mg citric acid per liter of sterile water) to prevent the oxidation of phenolic compounds which causes blackening of tissues.

Culture initiation and multiple shoot induction

The rhizome buds in antioxidant solution are taken to laminar flow chamber for sterilization and inoculation. The rhizome buds are sterilized using 70% ethanol for 30 sec followed by 0.12% mercuric chloride for 10 min. The rhizome buds are washed several times with sterilized distilled water to remove the traces of sterilants. The rhizome buds are finally trimmed to remove the outermost whole of tissue exposed to sterilants. A vertical cut is given to arrest the apical dominance and to induce adventitious buds. The rhizome buds are inoculated in initiation and multiple shoot induction media. The explants in media are incubated at 26-28° C with 12 hours light and dark alternating period. After 4-6 weeks, buds can he observed for growth.

Subculturing

The rhizome buds after 4-6 weeks are subcultured by trimming off the blackened tissues followed by vertically cutting into small pieces. Each explant is inoculated in initiation and multiple shoot induction media. After 6-8 weeks 5-7 multiple shoot can be observed from each explant. Single shoots are transferred with a small portion of rhizome to initiation and multiple shoot induction media. This step is repeated to 5-6 times to get required number to shoots.

Shoot elongation

The shoots are inoculated in shoot elongation medium for 2 weeks. If the shoots are already elongated during subculturing then the shoots are directly transferred to rooting media. Single shoots are cut from the multiple shoots and each shoot is inoculated in rooting media at the rate 5-6 shoots per bottle. Rooted shoots can be observed after 3-4 weeks.

Hardening

The plantlets in the bottles are washed to remove the agar. The plantlets are separated, sorted based on size, transplanted into boxes of soil (2:1:1 of sand: red soil: FYM) measuring 58x36x12.5cm. High planting density (126 plantlets/box) is maintained. After planting, the box is covered with plastic sheet for seven to ten days, to conserve moisture. Then the plants are transferred to plastic pots and maintained under sub-optimal conditions for 15-45 days.

Media

Initiation and multiple shoot induction: MS + BAP 5 mg /l

Shoot elongation: MS+ BAP 2mgll + IAA 0.5 mg/l

Rooting: ½ MS +IBA 0.5 mg/l +NAA 0.5mg/l+ 0.05% activated charcoal.

Ex:no 8 **Regeneration via direct organogenesis** **Page No:**

Date: **(Micropagation of Chrysanthemum)**

Chrysanthemum (Golden flower) is one of the common ornamental plants of world belonging to the family compositae. It is important not only for its outstanding aesthetic beauty & a long lasting capability but also because of its good potentials for marketing as cut flowers and potted plants to many comities. Chrysanthemum is available in all colors including bronze, lime green, brick red except blue. Chrysanthemum are savable in all color including bring, lime green, brick red except for blue.

Chrysanthemum are propagated vegetatively either through root suckers or terminal cuttings. This conventional process of shoot cutting is very slow. For commercial purpose, we need a large-scale production. Due to the high popularity & demand for chrysanthemum it becomes one of the first commercial targets for micropropagation. It is also known for its wide range of shapes and sizes.

Procedure of Direct organogeneis

1. Establishment of explant

The explants were excised from the mother or source plant using a sharp blade/knife and surface sterilized using 0.1% of any surface sterilizing agent followed by washing with sterile water for 4 to 5 times. Now the explants were ready for inoculation for shoot initiations.

2. Shoot initiation: The sterilized explants were inoculated in the shoot induction medium (SIM) which comprises of MS basal medium supplemented with different concentrations of cytokinin for shoot initiations.

3. Rooting initiation: The shoots were transferred to rooting initiation medium (RIM) which comprises of MS basal medium supplemented with different concentrations of auxins like NAA or IBA or IAA for root initiations.

4. Acclimatization (Hardening and field establishment):

The rooted shoots should be transferred to the pots (10 cm dia) containing a mixture of FYM: sand mixed in 1:1 ratio. Initially the plantlets should be covered with polythene covers to maintain high relative humidity. After 15 - 20 days, when they show initial signs of establishment, the covers can be temporarily removed daily for few hours and the plants should be sprayed with H2O. After four weeks, the covers can be removed completely and the plants can be kept in glass - house and gradually exposed to normal condition.

Protocol:

Take the explants (shoot tips/ nodal segments/petioles) from 4 months old plants

↓

Wash thoroughly under running tap and distilled water

↓

In laminar airflow cabinet, surface sterilize the plants with 70% ethanol (30 sec) followed by 0.1% $HgCl_2$ for five minutes

↓

Wash with sterile distilled water for 3-4 time

↓

Inoculate the explants in culture initiation medium

↓

Incubate at 25 ± 2°C and keep it under 14 hour photoperiod fluorescent tube light

↓

Transfer the explants after 4 weeks from culture initiation medium to multiplication medium

↓

Subculture at 3-4 weeks interval

↓

Separate the shoots & place it in shoot elongation medium

↓

Transfer the elongated shoots to rooting medium

↓

After rooting, wash the plantlets to remove the adhering agar and plant in potting mixture containing Sand: Red earth: FYM @ 2:1:1 for establishment

↓

Harden in mist chamber

Media

Culture initiation : MS medium with BAP 0.5 mg / L +0.8% agar
Multiplication media : MS medium with BAP 3mg / L +0.8% agar
Elongation media : MS medium with BAP 1 mg / L +0.8% agar
Rooting media : 1/2MS medium with IBA 1mg / L + 2% sucrose + 0.6% Agar

Work done

Ex:no 9 **Regeneration via indirect organogenesis** **Page No:**

Date: **(Micropagation of Gerbera)**

Gerbera (Transvaal Daisy / Barbeton Daisy) is a genus of valuable ornamental plants from the sunflower family (Asteraceae), grown as a potted plant as well as for its cut flowers. Gerbera is very popular and widely used as a decorative garden plant or as cut flowers. Nearly thousands of cultivars exist. They vary greatly in shape and size. Miniature gerbera's called, as "germinis" are also available. They are smaller and appropriate for small flower arrangements and offer an incredibly wide range of colors with every color except blue represented (including fashionable shade of buff and maroon). The center of the flower is sometimes black; often the same flower can have petals of several different colors. Gerbera is commercially important. It is the fifth most important cut flower in the world (after rose, carnation, chrysanthemum, and tulip). It is also used as a model organism in studying flower formation, and contains naturally occurring coumarin derivatives.

Propagation

Originally, growers depended on seed for propagation. Later, they switched to cuttings, but the technique considered best these days is tissue culture. With tissue culture, we cannot only grow good varieties, but also vigorous plants that produce long-lasting, high-quality flowers.

***In vivo* problems**

1. Since, the genetic variability within *Gerbera* genus is relatively limited, breeding potential for new flower colors and patterns as well as resistance to biotic or abiotic stresses is also limited.

2. The vegetative propagation through divisions of shoot tip culture is possible, however, plant multiplication by this method is too slow to be commercially viable.

Indirect organogenesis

Petioles and leaves can be used as explants for gerbera micropropagation. The leaves with petioles should be collected from aseptically grown cultures of gerbera plant. Both leaves & petioles should be cut into small pieces (5-6 mm) under sterile conditions. The plants are regenerated from the explants through **indirect organogenesis** through the formation of calli. The calli from leaf pieces will differentiate into shoots after six weeks of incubation on the medium for callus induction whereas the

calli from petiole explants will take only three weeks for the same. The shoots will turn into green when transferred to 16h photoperiod. We can get an average of 12- 15 shoots per callus.

1. Establishment of explant

The explants were excised from the mother or source plant using a sharp blade/knife and surface sterilized using 0.1% of any surface sterilizing agent followed by washing with sterile water for 4 to 5 times. Now the explants were ready for inoculation in medium for callus initiations.

2. Callus initiation: The sterilized explants were inoculated in the Callus induction medium (CIM) which comprises of MS basal medium supplemented with 2, 4 -D 2 mg L^{-1}. The cultures should be incubated in dark for 30 days and transferred to light (100 μ mol m^{-2} s^{-1}) with 16 h photoperiod. After 30 d the callus obtained were compact and light to brown in colour.

3. Shoot initiation: The calli were transferred to shoot induction medium (SIM) which comprises of MS basal medium supplemented with BAP (3mg L^{-1}). Rapid multiplication of the shoots can be attained in MS Medium containing BAP (1mg L^{-1}). for the formation of shoots.

4. Rooting initiation: The shoots were transferred to rooting initiation medium (RIM) which comprises of MS basal medium supplemented with Individual shoots can be separated and rooted on Ms medium supplemented with IBA (1.5mg L -1) + IAA (0.1 mg L-1).

5. Acclimatization (Hardening and field establishment):

The rooted shoots should be transferred to the pots (10 cm dia) containing a mixture of FYM: sand mixed in 1:1 ratio. Initially the plantlets should be covered with polythene covers to maintain high relative humidity. After 15 - 20 days, when they show initial signs of establishment, the covers can be temporarily removed daily for few hours and the plants should be sprayed with H2O. After four weeks, the covers can be removed completely and the plants can be kept in glass - house and gradually exposed to normal conditions.

Work done

Ex:no 10 **Regeneration via direct somatic embryogenesis** **Page No:**

Date: **(*Carica papaya* cv. Sunrise)**

Embryo formed directly from the explants

Papaya (*Carica papaya* L.) is an important and a very popular fruit crop of the tropical and subtropical countries of the world. The crop has a high food value and is sought by all classes of people for its medicinal properties. Demand of the crop among consumers is high because of its early bearing habit and as it produces fruit throughout the year. Papaya is grown all over tropics and subtropics regions. However, major papaya producing countries are Brazil, Nigeria, Congo, Indonesia, Malaysia and India. Papaya Ringspot Virus (PRSV) occurs in most of the papaya producing countries and is the most harmful one (Purcifull *et al.* 1985). The virus is probably the single threat in commercialization of papaya cultivation.

Protocols for somatic embryo induction in papaya have been developed for a variety of reasons ranging from interest in methods for mass propagation to a requirement for recipient tissues for gene transfer technology (Fitch and Mansherdt 1990; Fitch 1993). Ironically, somatic embryos were produced in cultures of papaya hybrid embryos, which resulted from crosses between PRSV resistant wild species and commercial papaya cultivars.

Protocol:

Plant materials (explant)

Immature seeds of *Carica papaya* obtained from 90-105 d-old fruits were used as source of explants. Immature zygotic embryos excised from the immature seeds were used as explants.

Somatic embryo induction

Immature seeds collected from 90-105 d old fruits were surface-sterilized with 0.1% $HgCl_2$ for 5 min and washed thrice with sterile distilled water. Embryos were excised aseptically under a microscope (Leica, Switzerland) and cultured on a MS (Murashige & Skoog, 1962:) medium (MMS) supplemented with 2, 4-D (15.0 mg l^{-1})**.** The cultures were incubated in dark at 25°C for 8 wk for embryo induction.

Shoot induction

The Zygotic embryo derived somatic embryos were transferred onto MS regeneration medium supplemented with Kinetin (5 mg l^{-1}) for shoot induction. The tissues were incubated for 3 month at 25 °C with a photoperiod of 16 hours with illumination of cool white fluorescent light, 35 $\mu Mm^{-2}sec^{-1}$ PAR, at 27 °C.

Root induction

Shoots of 2-3 cm height with 2-3 trilobed leaves were transferred to half MS medium and cultured for 30 d. Plants with roots of 1-2 cm were ready for hardening.

Hardening of plantlets

The rooted plantlets were transferred to sterile potting mixture (containing loam , sand and farmyard manure) treated with Agrason fungicides, moistened with liquid MS medium and maintained in greenhouse for hardening. Well established plants were planted in field.

Reference

Fitch, M.M.M. and Manshardt, R.M. 1990. Somatic embryogenesis and plant regeneration from immature zygotic embryos of papaya (*Carica papaya* L.). **Plant Cell Rep. 9:** 320-324.

Work done

Ex:no 11 **Regeneration via indirect somatic embryogenesis** **Page No:**

Date: **(*Carica papaya* cv. Co7)**

Somatic embryos formed from the calli derived from explants

Papaya (*Carica papaya* L.; Family, Caricaceae), originated in tropical America, is one of the most important fruit crops in tropical and subtropical regions of the world. It was an introduced crop to India from Philippines through Malaysia and its cultivation has now spread widely in the tropical plains of the country. Apart from fresh consumption, papaya fruits are widely used in commercial sector for the production of jam, syrup, preservatives, 'papain' enzyme and several pharmaceutical products. It is predominantly grown in India, Nigeria, Brazil, Indonesia, Mexico, Peru, Congo, Thailand, Venezuela, Philippines, Bangladesh, USA (Hawaii), Sri Lanka, Malaysia and Australia with an estimated production of 5.44 million tonnes from 3, 40,594 ha annually. Major constraints in papaya production are non-availability of healthy planting material due to the limitation in multiplication of propagule by invitro culture technique and crop losses due to pest and disease incidence. Traditionally papaya is propagated at large scale through seeds that showed considerable variation in commercial population (Litz & Conover, 1978). In such circumstances, in vitro propagation provides an essential means of multiplication of selected genotypes.

Protocol

Plant materials (explant)

Immature seeds of *Carica papaya* (Co7) obtained from 90-105 d-old fruits (collected from the Horticultural College and Research Institute, Tamil Nadu Agricultural University, Coimbarore) were used as source of explants. Immature zygotic embryos excised from the immature seeds were used as explants.

Callus induction

Immature seeds collected from 90-105 d old fruits were surface-sterilized with 0.1% $HgCl_2$ for 5 min and washed thrice with sterile distilled water. Embryos were excised aseptically under a microscope (Leica, Switzerland) and cultured on a modified MS (Murashige & Skoog, 1962:) medium (MMS) supplemented with 2, 4-D (2.0 mg l^{-1})**.** The cultures were incubated in dark at 25°C for 8 wk for callus induction.

Induction of somatic embryos

The embryogenic calli derived from zygotic embryo were transferred to half-strength MS medium. The culture was maintained for 3 months by sub culturing at regular intervals for induction and maturation of somatic embryos.

Shoot induction

The somatic embryos were transferred onto modified MS regeneration medium (MSR) supplemented with BAP (0.2, mg l^{-1}) and NAA (0.02 mg l^{-1}) for shoot induction. The tissues were incubated for 3 month at 25 °C with a photoperiod of 16 hours with illumination of cool white fluorescent light, 35 $\mu Mm^{-2}sec^{-1}$ PAR, at 27 °C.

Root induction

Shoots of 2-3 cm height with 2-3 trilobed leaves were transferred to modified MS medium (MSL) supplemented with IBA (1.0 mg l^{-1}), cultured for 30 d with frequent subculturing. Plants with roots of 1-2 cm were ready for hardening.

Hardening of plantlets

The rooted plantlets were transferred to sterile potting mixture (containing loam , sand and farmyard manure) treated with Agrason fungicides, moistened with liquid MS medium and maintained in greenhouse for hardening. Well established plants were planted in field.

Reference

Anandan, R., Pranathi, s., Balasuramanian, P. and Sudhakar, D.2007. An effective method for Somatic Embryogenesis and Plant Regeneration of papaya cv.Co7.**Recent Trends in Horticultural Biotechnology,** 26th Book Chapter, New India Publishing Agency, pp 267-269.

Work done

Ex:no 12 **Synthetic seeds** **Page No:**

Date:

Large scale production of somatic embryos and their encapsulation is referred to as Artificial or synthetic seed production. It is an alternative to traditional micro propagation for production and delivery of cloned plantlets. Artificial or synthetic seed is a bead of gel containing somatic embryo or shoot bud and the nutrients, growth regulators. Pesticides, antibiotics etc needed for the development of a complete plantlet from the enclosed somatic embryos or shoot bud.

Production of Synthetic Seed:

1. Explant is selected from choice of plant
2. In a laboratory using tissue culture techniques callus is induced in the explants.
3. Using tissue culture techniques somatic embryo is induced in the callus.
4. Somatic embryo are proliferated
5. Histodifferentiation and maturation of somatic embryo
6. Desiccation and tolerance induction using tissue culture techniques.
7. Encapsulation of somatic embryo
8. In vitro germination or transported to field for germination.

Artificial seeds may be produced by one of the two following ways

1. **Using desiccated embryos in coated condition**
2. **Hydrated condition, using sodium-alginate or calcium alginate hydrogels as the encapsulation material**
3. **Using polymer, self-breaking capsules releasing nutrient;**
4. **Using fluid drilling method.**

Desiccation method

In the dessicated system Kin and Janick (1989) developed the desiccated artificial seeds applying synthetic seed coats. They mixed equal volume of embryo suspension and 5% solution of poly-ethylene oxide, a water soluble resin, which subsequently dried to form strip containing multiple embryos. The embryos used here are desiccated by hardening treatments with 12% sucrose and using 10^{-6} M ABA followed by chilling.

Hydration methods

In the Hydrated systems, somatic embryos are enclosed in gels which remain hydrated. Of the many gels evaluated "calcium alginate is the most suitable. Artificial seeds can be made easily as follows. A 2% solution of Sodium alginate is filled in a burette and allowed to drip drop by drop into a 100 millimolar $Cacl^2$ solution. As the sodium alginate bead or drop forms at the tip of the burette, a somatic embryo is inserted into it with the help of a spatula before the drop falls into the Cacl2 solution. The beads become hardened as calcium alginate is formed. After about 20-30 min the artificial seeds are removed, washed with water and used for planting. Hydrated seeds are sticky and difficult to handle on a large scale and dry rapidly in the open air. These problems can be resolved by providing a waxy coating over the beads. Alternatively, a desiccated system may be use to produce a synthetic seeds. However it is not possible to store, except at low temperature and for a limited period hydrated artificial seeds and they have to be planted soon after they are

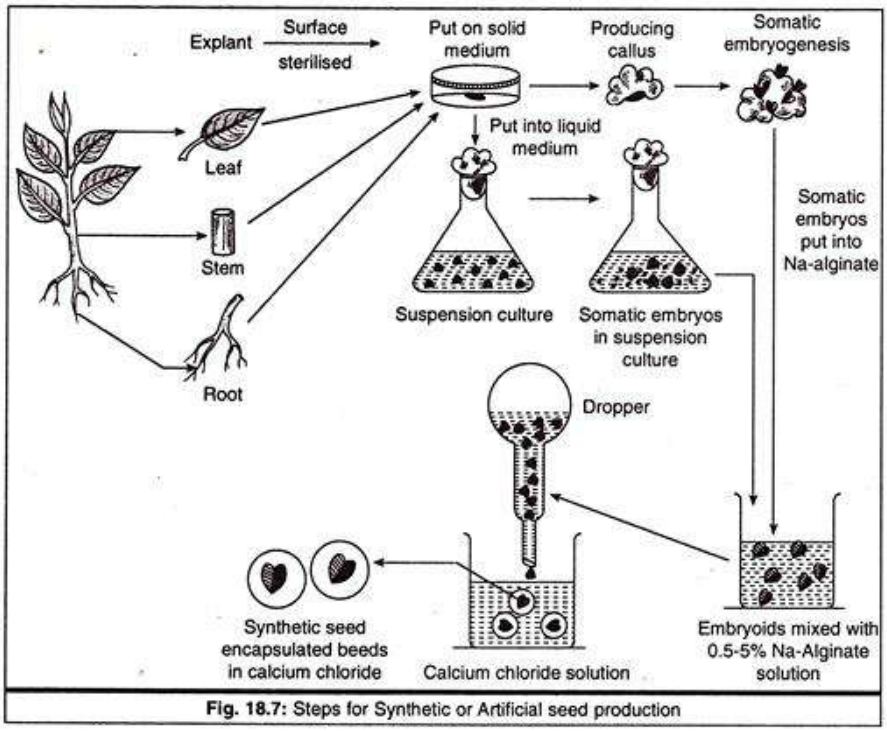


Fig. 18.7: Steps for Synthetic or Artificial seed production

Methods for artificial seed encapsulation

There are two methods for encapsulation listed as follows.

- **Dropping method**
- **Molding method**

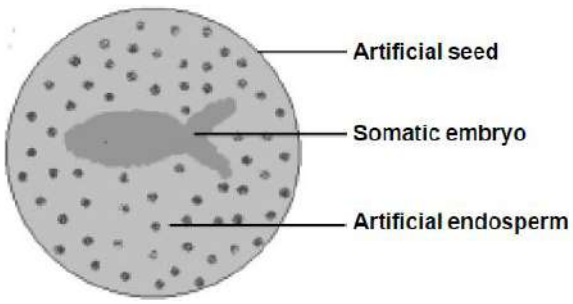
Procedure for dropping method

- Somatic embryos are dipped in hydrogel, this step encapsulate SEs.
- Hydrogel used may be any of the following.

  alginate – sodium alginate, agar from see weeds

  seed gums like guar gum, locust bean gum
- Here we have considered sodium alginate solution (1 – 5%), prepared in MS basal medium solution. SEs are dipped in this solution.
- These coated beads are added one by one with the help of pipette (5mm) into a complexation solution flask kept on magnetic stirrer and kept such for around 20-30 minutes. Here we considered calcium nitrate solution (100mM) as complexation solution.
- Embryos get covered by calcium alginate which is a stable complex due to ionic bond formation, become harder. Seeds become harder. Then gelled embryos are washed with water or MS basal medium.
- Alternatively, a burette is filled with sodium alginate solution (1 – 5%), dripped into a calcium nitrate solution (100mM) drop by drop. Somatic embryo is inserted into the drop formed at the burette tip. Sodium alginate drop along with SE falls into the solution of calcium nitrate.
- Useful adjuvants like growth regulators, herbicides, insecticides, fungicides and mycorrhizae such can be supplied to the SE while encapsulation along with the matrix.
- This method is applicable for embryo / auxiliary / apical / adventitious buds.

Synthetic seed



Molding method

•This method follows simple procedure of mixing of embryos with temperature dependent gel (e.g. gel rite, agar).

•Cells get coated with the gel at lowering of the temperature.

Ex:no 13 **Production of haploids** **Page No:**

Date:

The term haploid refers to those plants which contains gametophytic number of chromo¬somes. Anther/pollen culture mediated production of haploid plants approves the concept of totipotency. Guha and Maheshwari in 1964 reported direct development of haploid embryo from pollen of Datura innoxia by anther culture.

Anther culture procedure:

Step1

Experimental material: Young healthy plants grown under controlled conditions are used as experimental material from which flower bud of right stage (varies with species) is excised.

Step2

Disinfestation, excision and culture of anther: Flower buds are surface sterilized in laminar flow chamber followed by excision of anther from the bud. Stage of pollen development is determined by squashing an anther in acetocarmine and observing it under microscope. While excising anthers from flower buds, care is taken that anthers are not injured as injury leads to callusing hence giving mixture of diploids, haploids and aneuploids.

Step3

Culture medium conditions: The anthers are generally cultured on a solid agar medium where they develop into embryoids for anther culture under alternate light and dark period. Medium should have sucrose for induction of embryogenesis.

Step 4

Haploid plants: In species following direct **androgenesis** i.e. which develop through embryoid formation, small plants emerge in 3-8 weeks after culturing which are then transferred on to a rooting medium with low salt and small amount of auxin. Those species undergoing indirect androgenesis involving callus formation, callus is removed from the anther and placed onto a regeneration medium with suitable ratio of cytokinin to auxin. The haploid plants thus produced in both cases are transplanted to soil in small pots and maintained under controlled conditions in greenhouse.

Diploidisation of haploid plants: Haploid plants produced from anther culture maintained *in vitro* can grow till the flowering stage but cannot be perpetuated. Since these plants are haploid and have only one set of homologous chromosomes of the diploid species, they cannot form viable gametes and

hence no seed setting takes place for further perpetuation. Therefore, it is necessary to double the chromosome number of haploids to obtain homozygous diploids or dihaploid plants followed by their transfer to culture medium for further growth.

Application of haploid production:

Diploidisation of haploid plants result in rapid achievement of homozygous traits in doubled haplouds, hence these anther derived haploid plants have been used in breeding and improvement of crop species.

1. **Production of homozygous lines:**

The most important use of haploids is the production of homozygous lines which may be used directly as cultivars or may be used in breeding programme. For e.g. doubled haploids have been used for rapid development of inbred lines in hybrid maize programme.

The anthers from F1 hybrids of selected or desirable cross are excellent breeding material for raising anther derived homozygous plants or doubled haploids in which complementary parental characteristics are combined in one generation. The doubled haploid plants are subjected to selection for superior plants **(F**ig.2**)**. This approach is described as **hybrid sorting** where recombinant superior gametes are virtually being selected since the heterozygous gene combination in the F1 hybrid is transformed into homozygous combinations. Hybrid sorting reduces the time required for haploid breeding by 4-5 years as in conventional breeding by pedigree/ bulk method, the same requires ten years. Also, selection among DH lines reduces the size of breeding population.

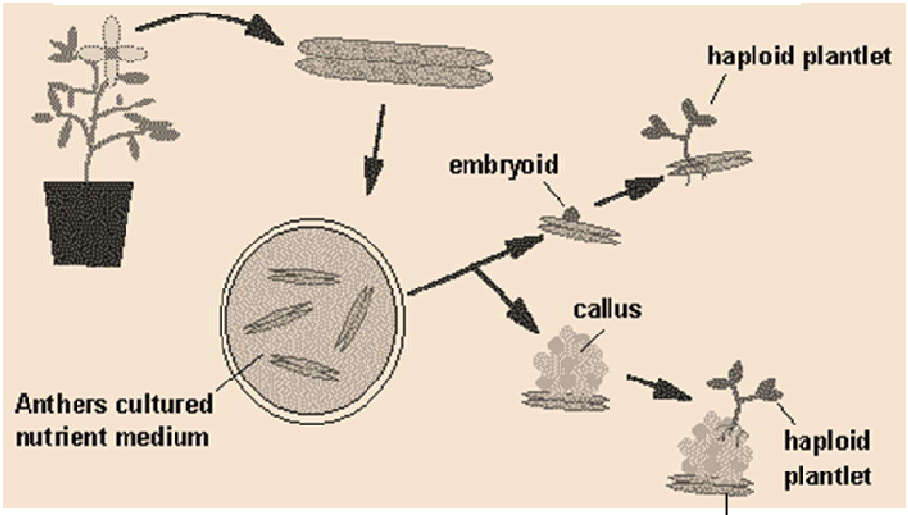
2. **Gametoclonal variation:** The variation observed among haploid plants having gametic chromosome number developing from anther culture is called gametoclonal variation. Such variations resulting in desirable traits are subjected to selection at haploid level followed by diploidisation to get homozygous plants which can be released as new varieties.

3. **Selection of desirable mutants:** Haploids offer a system where even recessive mutations are expressed unlike diploids where they express only in segregrating single plant progeny in M2 generation. Therefore, in several crops desirable mutants including traits like resistance to diseases, antibiotics, salts etc have been isolated from haploids derived from anther culture. For e.g. tobacco mutants resistant to black shank disease and wheat lines resistant to scab (*Fusarium graminearum*) have been selected and used as improved cultivars.

Problems associated with haploid plants:

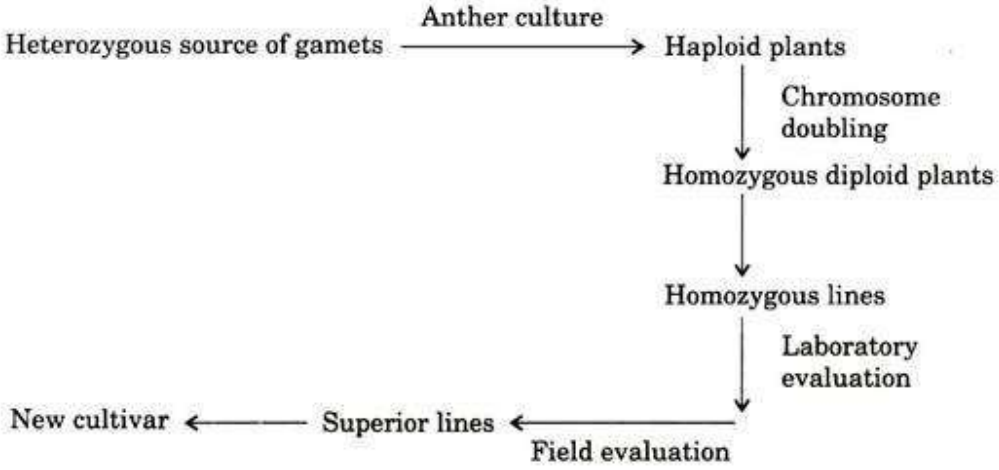
- • Many species are not yet amenable for haploid production
- • Deleterious mutations may be induced during *in vitro* phase.
- • Plants having more or lesser than gametic chromosome number is also obtained which necessitates cytological analysis first.
- • Occurrence of gametoclonal variation limits the use of anther derived embryos for genetic transformation

Regeneration of anther for haploid productions



Colchicines treatment for double haploids

Varietal development via anther culture



Ex:no 14 **Embryo culture** **Page No:**
Date:

Embryo culture

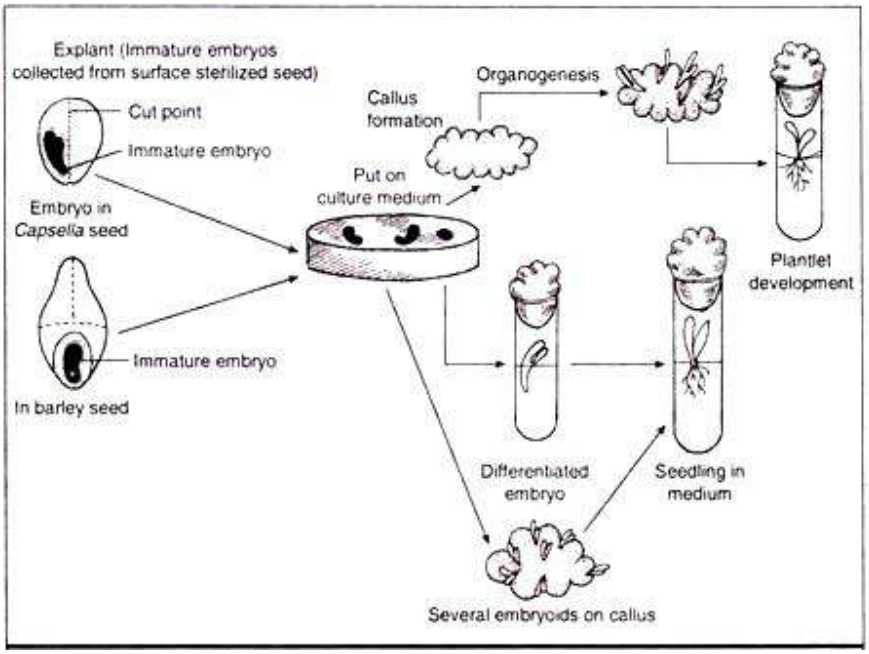
Besides, roots, shoots, and pollen, embryos can also be cultured to produce haploid plants. The embryo culture is very useful in conditions where embryo fails to develop due to degeneration of embryonic tissues. It has been used as a routine technique in orchid propagation, in breeding of species showing dormancy.

Methods of embryo culture

a. Embryo rescue

It has been observed that sometimes, inspite of successful pollination and fertilization, the embryos do not develop. The incompatibility between the embryo and the endosperm or some inherent deficiency also results in the under development of embryo. These immature embryos can be dissected out from the seeds and can be grown artificially on culture medium. These embryos differentiate into shoot, root and plantlets under culture conditions. This technique of growing immature embryo is termed as 'embryo rescue'. This technique is very useful in hybridization, breaking dormancy of certain seeds, and to achieve complete growth of embryo into a plant.

Procedure for embryo rescue



b. Embryo-nurse endosperm transplant

They inserted an excised hybrid embryo into a cellular endosperm dissected out from a normally developing ovule of one of the parents or a third species and cultured the nurse endosperm with the transplanted embryo on an artificial medium. Surface-sterilized pods carrying hybrid embryos or normally developing embryos (for nurse endosperm) were placed in sterile petri-dishes lined with moist filter paper until dissection. Two ovules, one carrying a hybrid embryo and the other with a normal embryo were removed from their pods and placed on a small (2 x 2 mm) pre-sterilized 'carrier' square of moist filter paper on the sterilized microscope stage.

Embryo transplant technique