A
Plant Biotechnology Laboratory Manual

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Introduction to Laboratory Practices

A laboratory is a workshop for a scientist. Here researcher does the techniques for preparation of chemical substances and formulate new methods. One must know what all procedures are involved in the experiment and what all types of equipment and chemicals are required for it. Proper knowledge about the working principles of the types of equipment and the nature of the chemicals are essential.

Instructions to Work in the Laboratory

1. Lab work is always totally different from any kind of office work. A researcher is not absolutely free from research thoughts at any time of the day. In that case, punctuality is of top priority.
2. Your attitude towards your work reflects the results you get.
3. Irresponsibility in any manner won't be tolerated.
4. Always keep your lab environment neat and tidy.
5. Chemicals, glassware and all lab belongings should be placed in the space provided.
6. Keep your working bench clean of everything. Never keep books, purses, bags, etc. on the working bench. Nothing should be lying on the bench.
7. Don't eat or drink or talk while working in the lab.
8. You must have a practical basic record, field book, a pen or pencil, a laboratory coat, a head cap, a mask, a lab slipper and a pair of gloves to work in the lab.
9. Record your results at time. For any difficulty, ask your laboratory in charge.
10. Record every single calculation in your field book and every step involved in the procedure.
11. Plan your work in order to finish it in stipulated time.
12. Be economical with reagents and other resources. Only small quantities of the reagents should be used.
13. Handle the glass equipment carefully. If it breaks report it to the lab in charge.
14. Dispose all the waste liquids in the sink; allow water to run for some time by opening the water tap.
15. Never spill any chemicals in or on the lab equipment. If so, clean the equipment soon after its use.
16. All the electric supplies must be plugged out if not in use.
17. Lights, fans, ACs and computer systems should be off if not required.
18. Water supplies should be closed tightly after use.
19. You should save electricity, water and gas at least for your future needs.
20. In case of any injury or burns go for a medical assistance with the first aid box provided in the laboratory.
21. Most importantly you should maintain a good relationship with your colleagues for their valuable support. Working with healthy groups always improve your scientific knowledge, skill and attitude.
22. You should be up to date with the recent trends and findings at least in your field of work.

You should record your work in the books (field book/lab book and basic record) provided. It is the property of the research station and hence you are not supposed to keep those books at home. When you resign from the job you should submit them up-to-date to the lab in charge without any delay.

Field Book/Lab Book

It is a rough record book and hence it should contain all the simple works related to the project. All the experiments conducted in the lab must be recorded in the book. It is a compilation of whole work done by

the researcher, so it must be well maintained. Also it can be a good reference book for those who come along.

You should note the following points while dealing with field book/lab book.

1. Keep the book neat and tidy.
2. Utilize the book efficiently preserving the legibility of your writing.
3. Name of the experiment should be entered along with the date of carrying out that experiment.
4. Next, you mention the requirements for the experiment.
5. Summarize the theory and principle. This should be followed by the procedure.
6. Mention the general calculations for the experiment. It should contain all the related works of the project for which it is meant for.

The following points are to be taken care of:

1. Do not tear pages from the field book. Number the pages of field book.
2. Do not over write if a mistake has been committed in recording, put a line over it and write the correct word again.
3. Complete the index, indicating the experiment, its serial number, page number on which it is written.
4. The notebook should always be up to date and may be collected by the lab in charge at any time.
5. You have to submit the field book and basic record at the end of every month on the date assigned.

Basic Record

1. Index: Provide an index containing the title of each experiment with page number and Sl.No.
2. Brief title of the experiment and date: Every experiment should have a descriptive title.
3. Aim: A clear objective should be there.
4. Technical Programme: This section should include any materials required, reagent composition, protocol and formulae. Procedure in the form of flow charts is helpful if it involves several parts. If an experiment is a repeat of an earlier experiment, you do not have to write down each step, but can refer to the earlier experiment by page or experiment number. If you make any changes, note the changes and reasons why.
5. Observations: Record periodical quantitative and qualitative observations
6. Result: This section should include the final result of the experiment in accordance with the aim. All raw data, including gel photographs, printouts, graphs, autoradiographs, etc if present are to be included.
7. Inference: The results obtained should be interpreted in accordance with the principle of the experiment.
8. Future Line: This section includes any suggestions from the protocol done, any refinements required etc.

It is mandatory to have clear and accurate records of all experiments conducted in the laboratory.

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Safety Measures and First Aid

Safety Measures

Exposure to several hazardous and toxic chemicals and other agents in a laboratory poses danger to the researcher so it is essential to adopt safety measures for their protection.

1. Prior to use equipment or a chemical the information and instructions should be read vividly.
2. It is essential to read the warning signs or labels on equipment and chemicals before using them. It is important to make sure that the location of the safety equipment like the eye washes, first aid kits, clean up kits and fire extinguishers is known along with the knowledge about their usage. All manufacturers of hazardous materials are required by law to supply the user with pertinent information on any hazards associated with their chemicals. This information is supplied in the form of Material Safety Data Sheets, or MSDS. MSDS information can be accessed on the web on the Biological Sciences Home Page. You are strongly urged to make use of this information prior to using a new chemical and certainly in the case of any accidental exposure or spill.
3. It is mandatory to wear lab coats, gloves, eye protection and inhalation protection masks when working with chemicals, UV light etc.
4. The volatile or potentially hazardous chemicals in a laboratory should be used in a fume hood only.
5. Providing safety hoods, good radioactive waste disposal systems, gloves when using hazardous carcinogenic chemicals and wearing of goggles for protection from UV light is essential.
6. In case of an injury, medical aid should be sought immediately.
7. A bottle should never be held by its neck, but instead firmly around its body, with one or both hands, depending on the size of the bottle to avoid spills.
8. Acids must be diluted by slowly adding them to water while mixing; water should never be added to concentrated acid to avoid splattering.
9. Acids, caustic materials and strong oxidizing agents should be mixed in the sink. This provides water for cooling as well as for confinement of the reagent in the event if flask or bottle breaks. Label the container before adding the reagent, and dispose of when proper expiry date is reached.
10. No eating, drinking or smoking in the lab.
11. Application of cosmetics is prohibited.
12. Wash hands frequently and hydrate with a good lotion.
13. Keep finger nails short.
14. At the end of the day clean all working benches with a disinfectant.
15. Tie back long hair.
16. Do not wear jewelry, loose or baggy clothing.

There are certain chemicals which are hazards and should be taken care of. They can be categorized as flammables, combustibles, explosives, oxidatives, toxic materials, compressed gases, corrosive materials, irritants and carcinogens.

Flammables: Substances which have a flash point or ignition point below room temperature. E.g. Oil, Gasoline, Ether etc. Storage rooms, cabinets and containers should be specially designed for such flammable liquids. Phenol can cause severe burns.

Combustibles: It is better to choose a combustible product over a flammable product if all other considerations are equal. Clearing agents offer this choice.

Explosives: Picric acid forms dangerous salts with certain metals which explode when wet. Avoid them altogether. Certain silver solutions, on ageing, explode by shaking. So never store these solutions after use.
Oxidatives: Oxidatives promote combustion in other materials but are harmless themselves. They have a risk of fire hazard when in contact with suitable material. E.g. Sodium iodate, Mercuric oxide, Organic peroxides.
Toxic materials: Causes death by ingestion, skin contact or inhalation, at certain specific concentration. E.g. Xylene and toluene are neurotoxins. Chloroform, Methanol, Xylene, Toluene are reproductive toxins, Acrylamide (potential neurotoxin), Formalin- toxic by ingestion and inhalation, Chromic acid, Osmium tetroxide and Uranyl nitrate are highly toxic.
Compressed gas: Gas at room temperature (20°C) and pressure, packaged as a pressurized gas by compression or refrigeration and is usually quite heavy. The potential hazard of compressed gases occurs when sudden rupturing of the container causes it to become a dangerous projectile. E.g. Propane & Acetylene bottles
Corrosive materials: Causes destruction of living tissue or irreversible alteration and destroy materials e.g. Bleach, Battery Acid, Ammonia & Hydrochloric Acid.
Irritants: Reversible inflammatory effects at the site of contact. Eyes, skin and respiratory passages are affected. Formalin is a skin and respiratory irritant.
Sensitizer: Causes allergic reaction. Sensitization lasts for life & gets worse with subsequent exposure. Formalin is a prime example.
Carcinogens: Ethidium bromide, Chloroform, Chromic acid, Dioxane, Formaldehyde, Nickel chloride, Potassium dichromate, certain dyes etc.

These chemicals are not harmful if used properly. Always wear gloves when using potentially hazardous chemicals, and never mouth-pipette them.

Also there are certain physical factors which require safe handling.

a. *Ultraviolet Light*: Exposure to ultraviolet (UV) light can cause acute eye irritation. Since the retina cannot detect UV light, you can have serious eye damage and not realize it until 30 minutes to 24 hours after exposure. Therefore, always wear appropriate eye protection when using UV lamps.
b. *Electricity*: You should take care of the electric circuits if there is any short circuit problem or anything like that. Always power off / unplug the equipment if not in use.
   Electrical equipment should not be handled with wet hands, nor should electrical equipment be used after liquid has been spilled on it. The equipment must be turned off immediately and dried thoroughly. In case of a wet or malfunctioning electrical instrument the plug should be pulled and a note of cautioning should be left on the instrument. Use of extension cords is prohibited.
   The voltages used for electrophoresis are sufficient to cause electrocution. Cover the buffer reservoirs during electrophoresis. Always turn off the power supply and unplug the leads before removing a gel.
c. General lab maintenance: Since you will use common facilities, all solutions and everything stored in an incubator, refrigerator, etc., *must be labeled*. In order to limit confusion, each person should use her/his initials or another unique designation for labeling plates, etc. Unlabeled material found in the refrigerators, incubators or freezers may be discarded. Always mark the culture/reagent bottles with your initials, the date, and relevant experimental data, e.g., concentration (mg/l).

First Aid

1) Chemicals in the Eyes: Getting any kind of a chemical into the eyes is undesirable, but certain chemicals are especially harmful. They can destroy eyesight in a very short time. If it does happen, remove lenses and flush your eyes with copious quantities of cool running water, for at least 20 minutes. The eyelid of any affected eye should be lifted up and the area beneath the eyelid irrigated as well. Seek medical treatment immediately.

Acid/Alkali splashes in the eye: Water spray from a wash bottle or rubber bulb into the medial corner of the eye. Put 4 drops of 2% Aqueous Sodium bicarbonate into the eye, if acid; and saturated solution of Boric acid, if alkali.

2) *Chemicals in the Mouth*: The chance of this kind of accident is unlikely. However, if it does happen, any chemical taken into the mouth should be spat out and rinse the mouth thoroughly with water. Many chemicals are poisonous to varying degrees. Note the name of the chemical and notify the teacher and office clinic immediately. If the victim swallows a chemical, note the name of the chemical and notify the lab in charge and office clinic immediately. If necessary, the office clinic will contact the Poison Control Center, a hospital emergency room, or a physician for instructions.

3*) Chemical Spills on the Skin*: Acid/Alkali splashes on the skin: Wash thoroughly; bath the affected skin with cotton wool soaked in 5% aqueous sodium carbonate if acid and 5% acetic acid or undiluted vinegar, if alkali.

For a small area, flush the skin with water first. For a small acid or base spill on the skin, neutralize an acid with baking soda; neutralize a base with boric acid. For a large amount of chemical slipped on the body, use the safety shower. For water spills on the floor wipe up excess water with paper towels. If necessary, use the water main valve to turn the water off.

Remove contaminated clothing and footwear. Care should be taken not to affect unexposed areas of the casualty, or yourself. Wash the affected areas with running water. The length of time that affected areas should be washed will vary depending upon the chemical, its hazards and characteristics. If unsure, wash the affected area for at least 20 minutes. Do not attempt to pick off any solid chemical contaminants that are attached to the skin. Cover the affected area with a sterile, non-stick dressing. If necessary, seek emergency medical treatment. Anyone who may be potentially exposed to a chemical requiring specific treatment, and local area first aid attendants, should be made aware of the specific treatments prior to the use of the chemical.

4*) Inhalation:* If a first aider is required to breathe for an unconscious casualty, a facemask should always be used. This provides a barrier and aids in preventing the inhalation or absorption of hazardous chemicals. The symptoms of a chemical exposure should be treated as appropriate, giving consideration to the product label, the Safety Data Sheet and any formal first aid instructions. Inhalation of certain chemicals can result in the onset of delayed pulmonary edema. These chemicals should be identified during the risk assessment stage.

Breathing Smoke or Chemical Fumes: All experiments that give off smoke or noxious gases should be conducted in a well-ventilated fume hood. This will make an accident of this kind unlikely. If smoke or chemical fumes are present in the laboratory, all persons even those who do not feel ill should leave the laboratory immediately. Make certain that all doors to the laboratory are closed after the last person has left. Since smoke rises, stay low while evacuating a smoke-filled room. Thoroughly ventilate the room before going back to work.

5) *Fire:* Fire in the laboratory may occur due to spirit lamps, electrical appliances or other inflammable reagents used in a laboratory. All laboratories should have a fire extinguisher and easy access to safety showers and fire blankets. For putting off the flames from the inflammable liquids, throw sand over it.

Severe burns: If the victim is on fire, roll him in a blanket or overall to smoothen the flames. Inform the physician. Lay the victim on the ground. Do not remove his clothing. Cover him if he is cold. Do not apply any treatment to the burns. This must be left to the physician.

Minor burns: Plunge the affected part into cold water or ice-water to soothe the pain. Apply Mercurochrome or Burnol ointment to the burn. Apply dry gauze dressing loosely. If the burn becomes infected or does not heal, refer the patient to physician. Never tear off the blisters that form over the burns. A person whose clothing or hair catches on fire will often run around hysterically in an unsuccessful effort to get away from the fire. This only provides the fire with more oxygen and makes it burn faster. It is the responsibility of the closest person to bring the fire blanket to the victim as quickly as possible. Smother the fire by wrapping the victim in the blanket.

6) Injury: Bleeding from a cut: Most cuts that occur in the laboratory are minor. For minor cuts, apply pressure to the wound with sterile gauze, wash with soap and water, and apply a sterile bandage. If the victim is bleeding badly, raise the bleeding part, if possible, and apply pressure to the wound with a piece of sterile gauze.

Injuries caused by broken glass*:* Wash the wound immediately to remove any glass pieces. Apply Mercurochrome or Burnol ointment to the wound. Cover with gauze and adhesive tape.

7) Fainting: If a person faints, lay the people down on the back. Position the head lower than the legs and provide fresh air. Loosen restrictive clothing.

8) Shock: People who are suffering from any severe injury (for example, a bad burn or major loss of blood) may be in a state of shock. A person in shock is usually pale and faint. The person may be sweating, with cold, moist skin and a weak, rapid pulse. Shock is a serious medical condition. Do not allow a person in shock to walk anywhere. While emergency help is being summoned, place the victim face up in a horizontal position, with the feet raised about 12 inches. Loosen any tightly fitting clothing and keep him or her warm.

Electric shock: The symptoms are fainting and asphyxia. Before doing anything else, put off the main switch. Send for a physician. Begin giving mouth to mouth respiration immediately.

9) Ingestion: Swallowing acid: Make the patient drink some 5% soap solution immediately. Make him/ her gargle with the soap solution. Give him/her 3 or 4 glasses of ordinary water. If the lips and tongue are burned by the acid, rinse thoroughly with water. Bathe with 2% aqueous Sodium bicarbonate.

Swallowing alkalies: Make the patient drink 5% solution of acetic acid or lemon juice or dilute vinegar. Make him gargle with the same acid solution. Give him 3 or 4 glasses of ordinary water. If the lips and tongue are burned by the alkali, rinse thoroughly with water; bathe with 5% acetic acid.

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Dos and Don'ts in the Lab

Important Precautions to be taken

1. **Do** plan and prepare for every laboratory exercise before coming to lab. Always do some homework before carrying out experiments in the lab.
2. Always be neat and tidy yourself when you work in tissue culture lab.
3. Lab trays should be properly labeled for its effective use. Never carry tissue culture lab trays outside unless for wash.
4. Do wear proper attire. Wear a lab coat, mask, head cap, sometimes gloves too.
5. Always sterilize all the items you deal in tissue culture lab including your coat, cap and mask.
6. Switch on all the lights and ACs in the culture room at 8.30 am and off by 4.30 pm giving eight hours incubation at 25°C.
7. Always keep the apparatus and all the lab wares needed by the side before start.
8. Keep work bench neat and clean before leaving the laboratory. Do wipe with detergents if necessary.
9. Dispose of biological materials such as microbes by sterilizing and in special containers for biological hazards. Do not throw other materials into these bags.
10. Dispose of broken glass and other sharp materials in the separate disposal boxes labeled "sharp materials".
11. Be sure where to dispose of hazardous chemicals.
12. Always use safety glasses or other eye protection.
13. Tie back long hair to avoid contamination and fire hazard.
14. Be very careful with Bunsen burners. Always keep the burner at distance from the organic solvents. Your sincere care will avoid fire accident. The burner must be turned off soon after the use.
15. Wash your hands before leaving the laboratory, even if you wore gloves, wash with disinfectant in running tap water before and after the work.
16. Locate eyewash stations, fire extinguishers and safety showers.
17. Be familiar with the chemicals in the laboratory and take maximum care in handling each chemical.
18. Keep chemicals back in position after use.
19. Leave coats, extra books, and personal items in your locker. Avoid tripping over these items and taking pathogenic, toxic, radioactive and other hazardous materials to home and your families.
20. Do clean LAF with ethanol (70%).
21. Wash all items inserted into the hood with 70% alcohol.
22. You must cut your nails regularly.
23. Always maintain aseptic condition while working with cultures.
24. After completion of work always label the cultures with names, code and date of work.
25. Tightly close all bottles and caps
26. Remove materials from the hood after work. *LAF is not a storage area***.**

1. **Don't** mishandle the chemical solutions, spirit lamp, UV light, instruments/apparatus or electricity.
2. Don't roam here and there in the laboratory without work or any aim.
3. Never misplace lab wares and other equipment.
4. Never leave the cultures in your LAF.
5. Don't eat, drink, smoke, chew gum or apply cosmetics in the lab. Never use a lab microwave, laboratory fridge or freezer for food.

6. Never eat or drink using lab glassware.
7. Don't touch any chemical with hand as some may be corrosive.
8. Don't leave a burner lit unless you are standing next to it because someone else might be injured if they do not realize it is lit.
9. Never taste a chemical as it may be poisonous.
10. Never use cracked or broken glassware.
11. Don't throw materials other than "sharps" into the "sharp materials" receptacles.
12. Don't place any chemical on hand.
13. Don't keep the reagent bottle open.
14. Never mouth pipette. This technique is very dangerous and will result in illness and death. If you used this method in the past, learn to use pipetting aids.
15. Don't wash solvents or hazardous materials down the drains. Don't combine chemicals for disposal unless you know they are not reactive with one another. Don't bring inflammable liquids such as alcohol near the flame.
16. Don't disturb the arrangement of reagents or chemicals in the shelf.
17. Don't spill out chemicals.
18. Don't keep water running if there is no use.
19. Don't put anything of the laboratory (e.g. pencil, thread, labels, inoculation needle, pins, etc) in your mouth, ears, nose and eyes.
20. Don't put your fingers in your eyes, ears, mouth while working in the lab.
21. Don't throw solid waste materials like filter paper pieces, test- tubes pieces in the sink.
22. Don't play with chemicals.

Equipment care

(*a*) *General Care.* Keep the lab equipment in good working condition. Don't use anything (any instrument) unless you have been instructed in its proper use. Report any malfunction immediately. Rinse out all centrifuge rotors after use, in particular if anything spills. Please do not waste supplies (chemicals). Use only what you need. If the supply is running low, please notify the lab in charge before it is completely exhausted. Occasionally, it is necessary to borrow a reagent or equipment from another lab; notify the lab in charge.

(*b*) *Micropipettes.* Most of the experiments you will conduct in the laboratory will depend on your ability to accurately measure volumes of solutions using micropipettes. The accuracy of your pipetting can only be as accurate as your pipette, and several steps should be taken to ensure that your pipettes are accurate and maintained in good working order. Then they should be checked for accuracy following the instructions given by the instructor. If they need to be recalibrated, do so. Since the pipettes will use different pipette tips, make sure that the pipette tip you are using is designed for your pipette.

(*c*) *Using a pH Meter.* Biological functions are very sensitive to changes in pH and hence, buffers are used to stabilize the pH. A pH meter is an instrument that measures the potential difference between a reference electrode and a glass electrode, often combined into one combination electrode. The reference electrode is often $AgCl_2$. An accurate pH reading depends on standardization, the degree of static charge, and the temperature of the solution.

(*d*) *Autoclave Operating Procedures.* Place all materials to be autoclaved on an autoclavable tray. All items should have indicator tape. The items should be properly covered with paper before placing in the autoclave. Separate liquids from solids and autoclave separately. Make sure the lids on all bottles are loose. The water level should be in between the yellow strips. All valves must be opened when it is on. When steam comes through the lower valve, close the valve and let the steam to pass the top chamber. As the steam comes out through the upper valve, close it. This makes the temperature and pressure to rise to 121^{o}C, 15lb pressure. Make sure the chamber pressure is at zero before opening the door.

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Plant Biotechnology Lab

Biotechnology Laboratory Requirements and Safety

The following facilities should be there in a Biotechnology laboratory.

1. Store room: for storing chemicals and glassware.
2. Cleaning and washing room: for general cleaning purposes.
3. General laboratory: for routine laboratory experiments.
4. Specialized rooms: preparation and sterilization room, laminar flow, sterile storage and culture rooms.
5. General instrumentation room: for PCR machine, gel documentation system, electrophoresis units, centrifuge, pH meter, balance, laminar flow, freezers, ice machine etc.
6. Proper disposal of media, cultures etc.

Fig.1. At Inoculation Room, Tissue Culturist Inoculating in Laminar Air Flow Chamber

A biology laboratory include good computer attached with a printer, autoclaves, sinks, water distillation units, deionizers, hot air ovens, radiation shields, incubators, temperature control or cold room, dark room for development of photographic films, -20^{o}C freezers, refrigerators, water baths, refrigerated centrifuges, micro centrifuges, trans illuminator with camera, scintillation counters to monitor radioactivity, blotting apparatus, hot plates, micro wave ovens, and many other miscellaneous items.

The laboratory needs a variety of glassware including reagent bottles, beakers, measuring cylinders, Erlenmeyer flasks, test tubes, pipettes, glass rods, petri dishes, and other culture vessels. Disposable sterile Petri dishes can be used for the culture of plant tissue. Other requirements include liquid nitrogen containers, syringes, needles, forceps, scalpels, membrane filters, magnetic stirrers, orbital shakers, nylon or nitrocellulose membranes, Para film, aluminium foil, marker pens, Whatman 3 M paper, ice bucket, latex gloves, plastic boxes, plastic bags and UV goggles, good quality chemicals, uninterrupted power supply and water supply.

Plant Tissue Culture Laboratory Organization

An ideal tissue culture laboratory should have at least two big rooms and a small room. One big room is for general laboratory work such as preparation of media, autoclaving, distillation of water etc. The other big room is for keeping cultures under controlled light, temperature and humidity. The small room is for aseptic work and for keeping autoclaved articles.

Fig. 2. Plant Tissue Culture Room

A tissue culture laboratory should be provided with the following equipment, lab wares and facilities:

1. *A Washing Area*: This is very important for a tissue culture laboratory. It should be provided with a large sink, running hot and cold tap water, brushes of various sizes, detergent and a bucket of single distilled water for a fine rinse of the washed glass goods. A number of plastic buckets are required for soaking the glass goods to be washed. Another separate bucket with lid is also required for disposing off the used or infected media before cleaning. Only this bucket should be kept outside of the room or cleaning area and should be cleaned twice in a week.
2. *Hot Air Oven*: For drying washed glass goods. (Details in chapter 5)
3. *Refrigerator*: Essential for storing various thermo labile chemicals like vitamins, hormones, amino acids, casein hydrolysate, yeast extract, coconut milk etc. Stock solutions of salts are also kept to prevent contamination.
4. *Distillation Unit*: Two big carboys are required for storing the distilled water.(Details in chapter 5)
5. *Weighing Balance*: Pan balance, chemical balance, electric balance and electronic balances
6. *pH Meter*: Adjustment of pH of the nutrient medium and solutions (Details in chapter 5)
7. *Autoclave*: Moist sterilization unit (Details in chapter 5)
8. *Working Tables*: Preparation of the medium
9. *Heater*: Needed for heating or warming the medium to dissolve agar or to melt the agarified medium.
10. *Microscopes*: Simple, compound, inverted binocular stereo dissection microscopes, some attached with camera and computer
11. *Glass Stands*: For keeping chemicals
12. *Inoculation Room*: This room should be without any window or ventilator in order to make it dust free. The rooms should be provided with double doors. The doors should have an automatic door closer. Before entering the room shoes should be kept outside. Also the room provided with air curtains if there is no double door.

    Laminar air flow cabinet: Horizontal
    - Maintains sterile working area
    - Air blows directly at the investigator
    - Enough to protect your cultures but not you
    - Air is recycled and goes through HEPA filter before being returned to the room

    It is the most suitable, convenient and reliable instrument for aseptic work. Longer period work is possible. Has a number of small blower motors to blow air which passes through a number of HEPA filters. Such filters remove particles larger than 0.3µm. The ultraclean air which is free from fungal and bacterial contaminants, flows at the velocity of about 27±3m/minute through the working area. All the contaminants are blown away by the ultra-clean air and thereby an aseptic environment is maintained over the working area. Before starting work, laminar air flow is put on for 10-15 minutes. The flow of air does not put out the flame of a spirit lamp. Therefore, a spirit lamp can be used conveniently during the work.
13. Culture Room: For incubation of cultures under controlled temperature, light and humidity. The culture room with double doors in order to make it dust free and to maintain a constant room temperature. One should enter the room keeping their shoes outside the door. Maintain the temperature 25±2^{o}C inside the culture room. Air coolers are used. Specially designed stand to keep culture vessels. Shelves made of glass or plywood. Flasks, bottles, jars, Petri plates can be placed directly. Each culture racks with fluorescent lamps which are photo periodically controlled by an automatic timer. A thermometer and a hygrometer are fixed on the wall at the safety corner of the room to check temperature and relative humidity, respectively. The relative humidity of the culture room is maintained above 50%.

Instruments

Table 1. Tools and Instruments used in a biotechnology laboratory

Instruments	Measurement in length (cm)	Size No.	Use
Scalpel with blade	12	Blade size:23	For cutting the explant or plant materials
Surgical scalpel	-	3	---do---
Forceps	25	-	For holding the material
Jewellery fine forceps	9	-	For peeling leaf epidermis
Arrow headed sharp needle	-	-	For dissecting out anthers
Spatula	12	-	For transferring or subculturing the material
Scissors	14	-	For cutting roots or shoots from aseptic culture

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Equipment: Principle and Working

Equipment Features

1. **Autoclave**

General Description: An autoclave is a large pressure cooker. It is a *moist sterilization* unit.

Principle: It operates with the principle of steam under pressure as the sterilizing agent. High pressures enable steam to reach high temperature, thus increasing its heat content and killing power. Most of the heating power of steam comes from its latent heat of vaporization (the amount of heat required to convert boiling water to steam).

Steam is able to penetrate objects with cooler temperatures because once the steam contacts a cooler surface; it immediately condenses to water, producing a concomitant 1, 870 fold decrease in steam volume. This creates negative pressure at the point of condensation and draws more steam to the area. A condensation continues so long as the temperature of the condensing surface is less than that of steam; once temperatures equilibrate, a saturated steam environment is formed. Achieving high and even moisture content in the steam-air environment is important for effective autoclaving. The ability of air to carry heat is directly related to the amount of moisture present in the air. The more moisture present, the more heat can be carried, so steam is one of the most effective carriers of heat. Steam therefore results in the efficient killing of cells and the coagulation of proteins. Moist heat is thought to kill microorganisms by causing coagulation of essential proteins. Another way to explain this is that when heat is used as a sterilizing agent, the vibratory motion of every molecule of a microorganism is increased to levels that induce the cleavage of intramolecular hydrogen bonds between proteins. Death is therefore caused by an accumulation of irreversible damage to all metabolic functions of the organism. Death rate is directly proportional to the concentration of microorganisms at any given time. The time required to kill a known population of microorganisms in a specific suspension at a particular temperature is referred to as thermal death time (TDT). All autoclaves operate on a time/temperature relationship; increasing the temperature decreases TDT, and lowering the temperature increases TDT.

Fig. 3. Autoclave

Standard temperatures/pressures employed are 115°C/10 p.s.i., 121°C/15 p.s.i., and 132°C/27 p.s.i. (psi=pounds per square inch).

Working: Steam enters the chamber jacket, passes through an operating valve and enters the rear of the chamber behind a baffle plate. It flows forward and down through the chamber and the load, exiting at the front bottom. A pressure regulator maintains jacket and chamber pressure at a minimum of 15 psi, the pressure required for steam to reach 121°C (250°F).Overpressure protection is provided by a safety valve. The conditions inside are thermostatically controlled so that heat (more steam) is applied until 121°C is achieved, at which time the timer starts, and the temperature is maintained for the selected time.

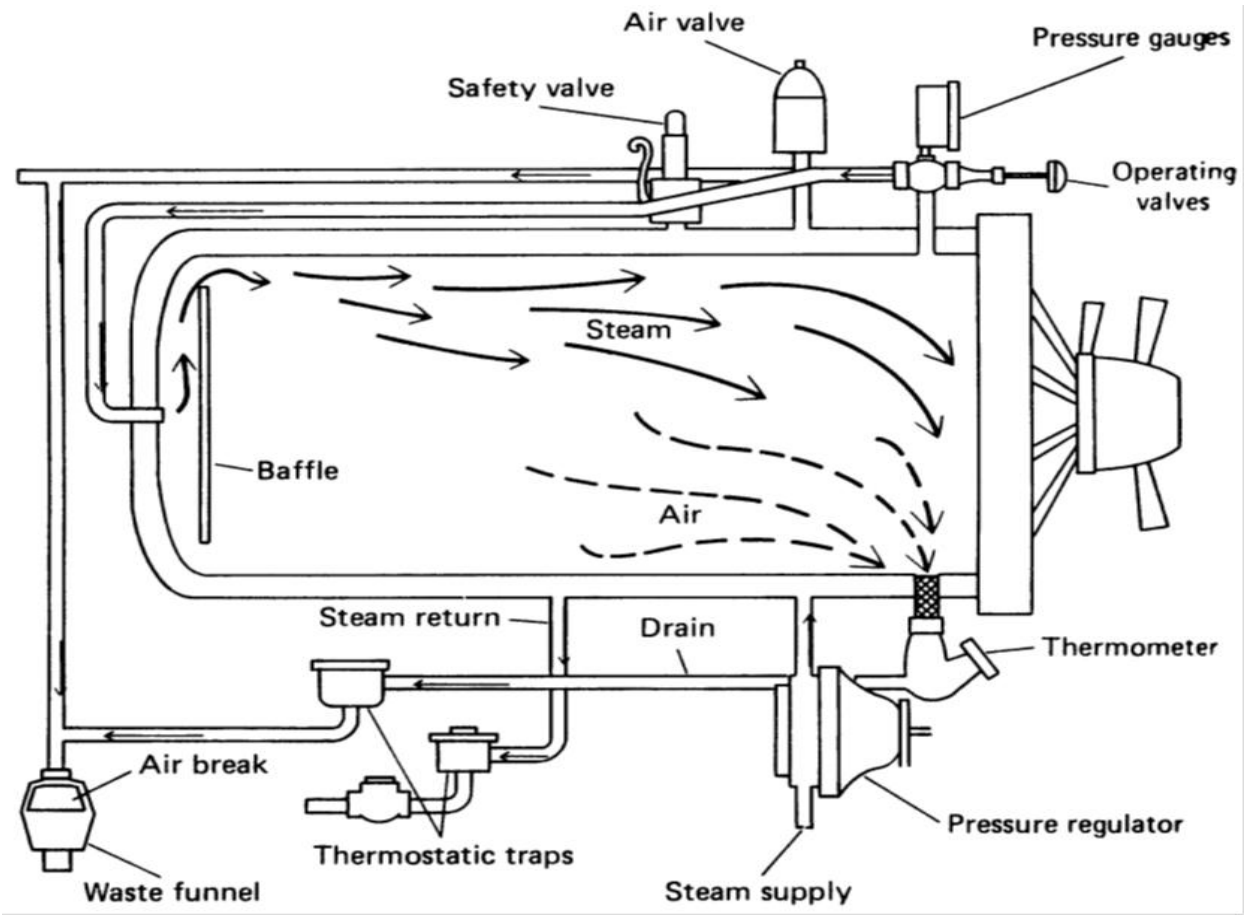


Fig. 4. Autoclave a detailed view

Points to Remember: Please note that after loading and starting the autoclave, the processing time is measured after the autoclave reaches normal operating conditions of 121°C (250°F) and 15 psi pressure, not simply from the time you push the "on" button. Due to the fact that autoclaves utilize steam, heat and pressure the risk of personal exposure and potential harm is great. Worker should wear proper personal protective equipment, i.e. heat resistant gloves, eye protection and a lab coat, particularly when unloading the autoclave. Regularly inspect the autoclave for proper operation. Do not assume that the temperature and pressure is down before opening the chamber. Look at the gauges. Even if the pressure gauge shows "0", open the chamber carefully; crack the door to allow steam to dissipate (don't fling the door open, as steam might come out and burn you). After opening the door, let items sit for five minutes before handling. This will reduce the chance of boil-over and burns. Never place sealed containers in an autoclave they might explode. This allows for expansion during the cycle. Caps must be slightly loose so that pressure created during the cycle does not cause the vessel to break. For screw-cap containers, you can make the lid hand tight and then loosen the lid by one-half turn. Always leave a few inches of "head room" in your containers. That way, if the item boils, it won't spray out into your face. Liquids to be autoclaved must be in an autoclavable vessel that is at least twice as large as the volume to be autoclaved (i.e. If you are autoclaving 1 liter of media, you need to put it in a flask that hold at least 2 liters). Always autoclave the media in a pan to contain spills. Agar will clog the drain in the autoclave and break it.

Do not autoclave items containing solvents, volatile or corrosive chemicals (phenol, trichloroacetic acid, ether, chloroform, etc.) or any radioactive materials.

2. Hot Air Oven

General Description: It is a *dry heat sterilization* unit. A dry heat cabinet is easy to install and has relatively low operating costs; it is nontoxic and does not harm the environment and it is noncorrosive for metal and sharp instruments.

Principle: Sterilization by dry heat is accomplished through conduction. The heat is absorbed by the outside surface of the equipment, and then passes towards the centre of it, layer by layer. The entire system will eventually reach the temperature required for sterilization. Dry heat does most of the damage by oxidizing molecules. The essential cell constituents are destroyed and the organism dies. The temperature is maintained for almost an hour to kill the most difficult of the resistant spores. The most common time-temperature relationships for sterilization with hot air sterilizers are:
170°C (340°F) for 60 minutes, 160°C (320°F) for 120 minutes, and 150°C (300°F) for 150 minutes or longer depending upon the volume.

Working: Working Principle of Hot Air Oven is the forced circulation of hot air inside the chamber of oven. As it is a universal scientific fact that in any chamber hot air rises above, so by utilizing this principle when the hot air reaches the top of chamber it is circulated back to bottom by a fan installed inside the chamber and hence optimum amount of heat is achieved gradually inside the Hot Air Oven.

Fig. 5. Hot Air Oven

Points to Remember: Dry heat sterilization technique requires longer exposure time (1.5 to 3 hours) and higher temperatures than moist heat sterilization. Dry heat ovens are used to sterilize items that might be damaged by moist heat or that are impenetrable to moist heat (e.g., powders, petroleum products, sharp instruments).

The Hot air oven is mounted on four rubber foots to prevent slipping and this protects the bench surface. The control panel houses a main ON/OFF switch indicator lamp and temperature setting knob. The scale is calibrated in 5°C steps.

3. Water Still

General Description: In this equipment liquid is vaporized (turned to steam), recondensed (turned back into a liquid) and collected in a container.

Principle: The separation of components from a liquid mixture *via* distillation depends on the differences in boiling points of the individual components. Also, depending on the concentrations of the components present, the liquid mixture will have different boiling point characteristics. Therefore, distillation processes depends on the vapour pressure characteristics of liquid mixtures. A liquid boils when its vapor pressure equalizes with the surroundings.

Fig. 6. Water Still

4. pH Meter

General Description: This equipment measures pH of the solutions and the tissue culture media. Appropriate buffers stabilize the pH of the electrode and the machine reads the pH of the solution in complement with the buffer.

Principle: Buffer is a solution whose pH does not change very much when small amounts of acid (H^+) or base (OH^-) are added. This does not mean that no change occurs, only that it is small compared to the amount of acid or base added; the more acid or base added, the more the pH will change. Buffer solutions consist of a conjugate acid-base pair (weak acid plus its salt or weak

Fig. 7. pH Meter

base plus its salt) in approximately equal amounts (within a factor of 10). Thus, buffers work best at pH within 1 pH unit of the pKa. The concentration of a buffer refers to the total concentration of the acid plus the base form. The higher the concentration of the buffer, the greater its capacity to absorb acid or base. Most biological buffers are used in the range of 0.01–0.02 M concentration. The ratio of the 2 components and the pKa of the acid component determine the pH of the buffer.

$$pH = pKa + \log[\text{base form}]/[\text{acid form}]$$

If everything is behaving ideally, the pH should not depend on the buffer concentration or the presence of other ions in solution. In reality, some buffers do better at this than others. It's best to check the pH of the final solution when preparing buffers from concentrated stocks. Temperature will also affect pH since pKa values, like other equilibrium constants, change with temperature. Again, it's best to check the pH of the buffer at the temperature it will be used.

The most commonly used electrode is made from borosilicate glass, which is permeable to H^+, but not to other cations or anions. Inside is a 0.1 M HCl solution; outside there is a lower H^+ concentration; thus the passage of H^+ from inside to the outside. This leaves negative ion behind, which generates an electric potential across the membrane.

$$E = 2.3 \times RT/F \times \log [H^+]_1/[H^+]_2$$

where R = gas constant, T = absolute temperature, F = Faraday constant, $[H^+]_1$ and $[H^+]_2$ are the molar H^+ concentrations inside and outside the glass electrode.

A reference electrode (pH-independent and impermeable to H^+ ions) is connected to the measuring electrode. Reference electrode contains Hg-Hg_2Cl_2 (calomel) paste in saturated KCl. The concentration of 0.1 M HCl (inside the measuring electrode) may decrease by repeated use. Therefore the pH meter has to be standardized against a solution of known pH.

Working:

- Turn on pH Meter. Lift up the electrode and clean the electrode tip by pressing with tissue paper.
- Calibrate using buffer 4±0.01 and buffer 7±0.01
- The buffers should come to the room temperature before calibration.
- Place the electrode in the solution to know the pH.
- If there is an increase in pH, stabilize it with adding 0.1N HCl which lowers the pH. If there is a decrease in pH, stabilize it with adding 0.1N NaOH which increases the pH.
- Add acid or alkali drop by drop and always stir well the solution with glass rod after each addition.
- Read the pH when √A appears.

Points to Remember:

1) Always keep the electrode dipped in buffer 4. Never leave the electrode dry.
2) Be cautious about the electrode level displayed on the screen. It symbolizes the fitness of electrode. Any kind of unusual appearance can cause pH fluctuation.
3) Buffer solutions should always be clear without any turbidity or mycelia growth. Same is the case if the buffer is used to protect electrode.
4) Always prepare fresh buffer solutions every month for calibration.
5) Always prepare buffer solutions in sterile water in the same method mentioned in buffer capsule box.
6) Never mishandle the electrode like using it for stirring the solutions.
7) Always use sterile water to clean the electrode after dipping it in solutions.
8) Never place the electrode in solutions which are in extreme temperature conditions.
9) No hurry should be there for reading the pH. Be patient while taking stable pH readings.
10) Do not forget to make entry with appropriate comments in the log book.
11) The equipment and its premises should be clean if there is any spill.

5. Electronic Weighing Balance

Principle & Working: Electronic weighing balance accurately measures the weight. Calibrate the balance by internal calibration. Place the weighing boat and tare the weight. Wait till it becomes zero. Chemical should be weighed slowly according to the need. Wait till the symbol "g" stabilizes next to the weight shown.

Points to Remember: Always have the knowledge about the maximum and minimum quantity which can be weighed using the balance. Never spill the chemicals on the weighing pan, if it happens wipe off with tissue. Switch off fan and windows nearby when working with it as it may cause fluctuations in the value due to interaction with air density. Always use weigh boats or butter paper to weigh.

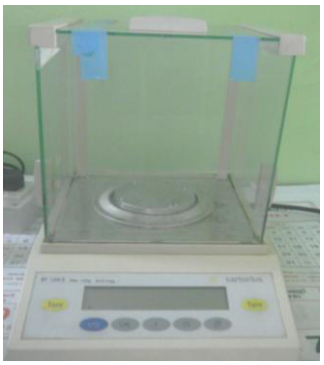


Fig. 8. Electronic Weighing Balance

6. Laminar Air Flow Chamber

General Description: It provides clean air to the working area, a constant flow of air out of the work area to prevent room air from entering. The air flowing out from the hood suspends and removes contaminants introduced into the work area.

Principle: The most important part of a laminar flow hood is a high efficiency bacteria-retentive filter. Room air is taken into the unit and passed through a pre-filter to remove gross contaminants. The air is then compressed and channeled up behind and through the HEPA filter (High Efficiency Particulate Air filter) in a laminar flow fashion; that is the purified air flows out over the entire work surface in parallel lines at a uniform velocity. The HEPA filter removes nearly all of the bacteria from the air.

Working: Switch on. Let the blower and light on. Wipe with 70% spirit in a vertical manner. Place all the materials required to work in the hood. All should be Spirit sterilized before placing in the LAF. Switch on the UV light for 45 minutes. Let the blower run continuously for 15 minutes. When this time has passed repeat the wipe out of the sterile area with an alcohol soaked piece of absorbent cotton. For cutting explants use a Petri dish (made of glass) or sterile steel plate which should be cleaned with an alcohol soaked piece of absorbent cotton.

Points to Remember:

- A direct path must be maintained between the filter (99.99% at 0.3 microns) and the area inside the hood where the manipulations are being performed. Air downstream from non-sterile objects (such as solution containers, hands etc.) becomes contaminated from particles blown off these objects.
- The hands should never obstruct airflow around the area where the needle enters the vial or culture bottle or culture. Also, when pulling the forceps, the fingers should not come in contact with any part of the culture.
- Always minimize time lag: Waste and other items should never enter the hood. All calculations should be done before entering the hood.
- Wash hands and arms before compounding or re-entering the hood. Also, remove any jewelry from the hands and wrists. It is important that you keep your hands within the cleaned area of the hood as much as possible. Do not touch your hair, face or clothing.
- Excess dust should be removed from items before introducing them into the hood.
- Arrange objects in a manner to get full benefit of the laminar flow of air. Critical items should be placed as close to the air source as possible. In a horizontal hood, items should be placed no closer than 3 inches from the very back of the hood (nothing should touch the filter). Occasionally, you may stack a few items however they must be stacked from lower to higher starting from the back of the hood.
- When working in a horizontal laminar flow hood, all work must be performed at a distance of no less than 15 cm (6 inches) from the front edge of the work surface. At a distance of less than 15 cm, laminar flow

air begins to mix with the outside air and contamination is possible. Never become so engrossed in your work that you forget this basic rule.

- Avoid spraying or squirting solutions onto the HEPA filter. Always aim away from the filter when opening cultures.
- Outer pouches and wraps should be removed at the edge of the work area as the sterile contents are pulled into the work area. Never bring these items into the main work area.
- Large objects should never be placed near the back of the hood. Not only do these objects contaminate everything downstream, but they also disrupt the laminar flow pattern of air which normally suspends the contaminants and removes them from the area.
- Remember that hand cleanliness is further reduced each time more bottles and other non-sterile items are handled.
- Before and after inoculation and in intervals, the work surface of the laminar flow hood should be thoroughly cleaned with 70% alcohol. A long side to side motion should be used starting at the back of the hood and then working forward. The acrylic plastic sides should also be cleaned periodically.
- It is possible to overcome the established airflow velocity by a strong reverse current produced by coughing, quick movements, talking etc. Keep all of these to a minimum in order to maintain a sterile environment. Do not talk, cough or sneeze into the hood.

7. Orbital Shaker

General Description: This is mainly used to provide for gentle and intensive mixing of biological and chemical compounds in a laboratory.

Working: It is a table-top laboratory instrument applicable for extracting, dissolving slow-reacting samples; cultivation of cells; extraction of mineral oil of soil, of tissue culture for analytical diagnostics; de-aeration of tested biodegradable materials and samples; rotating closed containers for dialysis.

Fig. 9. Orbital Shaker

8. Centrifuges

General Description: A centrifuge is a device for separating particles from a solution according to their size, shape, density, viscosity and rotor speed. In biology, the particles are usually cells, subcellular organelles, viruses, large molecules such as proteins and nucleic acids.

Principle: It works on the basic theory of sedimentation. Molecules separate according to their size, shape, density, viscosity, and centrifugal force. The simplest case is a spherical molecule. If the liquid has the density of "do" and the molecule has a density of "d", and if d>do, then the protein will sediment. In gravitational field, the motor force (Pg) equals the acceleration of gravity (g) multiplied by the difference between the mass of the molecule and the mass of a corresponding volume of medium.

The single most important advance in the use of centrifugal force to separate biologically important substances was the combination of mechanics, optics, and mathematics. Rotors for a centrifuge are either fixed angles, swinging buckets, continuous flow, or zonal, depending upon whether the sample is held at a given angle to the rotation plane, allowed to swing out on a pivot and into the plane of rotation, designed with inlet and outlet ports for separation of large volumes, or a combination of these.

Fixed angles generally work faster; substances precipitate faster in a given rotational environment, or they have an increased relative centrifugal force for a given rotor speed and radius. These rotors are the workhorse elements of a cell laboratory, and the most common is a rotor holding 8 centrifuge tubes at an angle of 34°C from the vertical.

In a centrifugal field, the gravitational acceleration (g) is replaced by the centrifugal force. Ultracentrifugation is carried out at speed faster than 20,000 rpm.

Super speed ultracentrifugation is at speeds between 10,000 and 20,000 rpm. Low-speed centrifugation is at speeds below 10,000 rpm.

Working: Cooling Centrifuge

Fig. 10. Cooling Centrifuge

•Switch on the mains. The power indicator will get illuminated.
•Setting the Program Number: Press the PROG button. The required program number can be set by using the UP or DOWN.
•Setting the timer: Press the PROG button again. The display panel will show Zero and the timer setting is initialized. The required time setting can be made by pressing the UP arrow for the increment and DOWN arrow for decreasing the time. Set the time required for the program and release the button.
•Setting the temperature: Press the PROG again. Now the system is ready for temperature setting. Set the required temperature by pressing the UP or DOWN arrow till the required temperature is set. Release the button.
•Selection of RPM: Press the PROG button again. The option to select the display mode to read normal display in RPM\RCF can be selected by using the UP or DOWN
•Setting the rotor speed: Press the PROG button again. Use the UP or DOWN arrow to set the required speed. Release the button
•Setting the acceleration time: Press the PROG button again. Use UP or DOWN arrow to set the acceleration time within the range of 60s-240s. Release the button.
•Setting the deceleration time: Press the PROG button again. The system is ready to set the deceleration time. Use UP or DOWN arrow to set the deceleration time within the range of 45s– 240s. Release the button.
•Rotor Selection: Press the PROG button again. The system is ready to set the rotor Selection. The Rotor heads are numbered and select the specific rotor head number which you plan to use in this program. Use arrow UP or DOWN select the specific number of the Rotor head you plan to use. Release the button, saving the setting and locking the program.

Now you have completed the setting of the parameters for the first program and the same is automatically registered and saved as parameters of the set program number as program 1. Press the RETURN key to come back to Normal Display mode.

Working: Microfuge

Fig. 11. Microfuge

- Choose a flat area on your table
- Put the line chord into a suitable 220v 50Hz power point
- Timer can be set in pulse mode. Timed cycles from 1-15 minute in increment of 1 minute and continuous mode
- The RESET—OFF key is to be pressed whenever time or rpm setting is to be changed.
- An audible beep sounds at the beginning and on completion of the cycle

Points to Remember:

1. After centrifugation wipe the inner chamber and keep open to be dried.
2. Clean the rotor after use. There are chances for any spill of liquids used.
3. Notice if any voltage fluctuation occurs.
4. It shows drive fault if any error occurs. In that case switch off and then enter the program.
5. Always make sure the required temperature is attained before the rotor starts.

9. Magnetic Stirrer with Hot Plate

Fig. 12. Magnetic Stirrer with Hot Plate

General Description: It is used to dissolve certain slowly dissolving chemicals and for mixing the solution. It is used in many biological labs.

Principle & Working: A magnetic stirrer is a laboratory device consisting of either a rotating magnet or stationary electromagnets creating a rotating magnetic field. This device is used to cause a stir bar immersed in a liquid to spin very quickly, agitating or mixing the liquid. A magnetic stirrer often includes a provision for heating the liquid. They

are preferred over gear-driven motorized stirrers because they are quieter, more efficient, and have no moving external parts to break or wear out (other than the simple bar magnet itself).

Due to its small size, a stirring bar is more easily cleaned and sterilized than other stirring devices. Magnetic stirrers avoid two major problems with motorized stirrers. Firstly, motorized stirrers use lubricants, which can contaminate the reaction vessel and the product. Secondly, in motorized stirrers, the sealing of the connection between the rotating shaft of the stirrer and the vessel can be problematic, especially if a closed system is needed.

Magnetic stirrers also have drawbacks. For example, the limited size of the stirring bar means it can only be used for relatively small (under 4 liters) experiments. In addition, viscous liquids or thick suspensions are extremely difficult to mix using this method, although there are some stirrers with special magnets to overcome this problem.

10. PCR Machine

General Description: A device which carries out polymerase chain reaction.

Principle & Working: The polymerase chain reaction (PCR) is an enzymatic process that allows for the detection of specific genes within an environmental DNA sample. PCR utilizes short, user defined DNA sequences called oligonucleotide primers, the sequence of which are complementary to target regions of genes known to encode for specific functions (e.g. contaminant degradation). In brief, the DNA sample is denatured to produce single stranded DNA, called template DNA, to which the oligonucleotide primers can bind. The enzyme DNA polymerase then adds nucleotide bases to the end of each primer, using the template DNA as a guide to extend the primer thereby producing new double stranded DNA. This process is repeated for a number of cycles to enrich the DNA sample for the desired genes targeted by the oligonucleotide primers. Since each cycle of PCR involves creating two new double stranded DNAs from each DNA molecule present, the amount of DNA theoretically doubles with every cycle of PCR. Therefore, after two cycles the concentration of DNA increases by 22-fold, after 3 cycles a 23-fold increase, etc. After N cycles, PCR generates a 2N-fold increase in the target DNA.

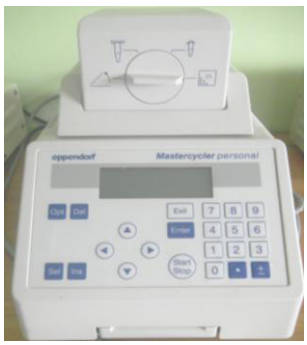
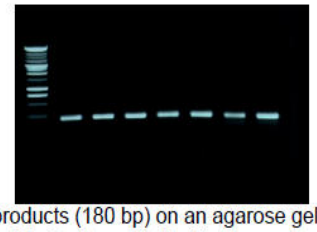


Fig. 13. PCR Machine


PCR products (180 bp) on an agarose gel. A DNA ladder is shown in the first lane

11. Electrophoresis Units

Electrophoresis is the migration of charged molecules in response to an electric field. Their rate of migration depends on the strength of the field; on the net charge, size and shape of the molecules, and also on the ionic strength, viscosity and temperature of the medium in which the molecules are moving. As an analytical tool, electrophoresis is simple, rapid, and highly sensitive. It is used analytically to study the properties of a single charged species, and as a separation technique. There are a variety of electrophoretic techniques, which yield different information and have different uses. Generally, the samples are run in a support matrix, the most commonly used being agarose and polyacrylamide. These are porous gels, and under appropriate conditions, they provide a means of separating molecules by size.

These can be denaturing or non-denaturing. Non-denaturing methods allow recovery of active proteins and can be used to analyze enzyme activity or any other analysis that requires a native protein structure. Two commonly used techniques are sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF). SDS-PAGE separate proteins according to molecular weight and IEF separates according to isoelectric point.

Agarose Gel Electrophoresis Unit

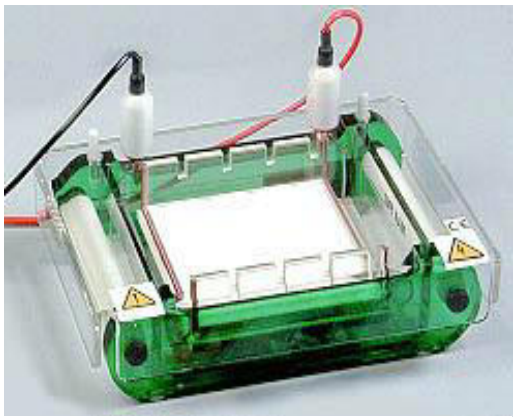


Fig. 14. Agarose Gel Electrophoresis Unit

General Description: Agarose gel electrophoresis is the horizontal gel electrophoresis which is the easiest and most popular way of separating and analyzing DNA. It separates the DNA fragments based on their molecular weight.

Principle: Here DNA molecules are separated on the basis of charge by applying an electric field to the electrophoretic apparatus. Shorter molecules migrate more easily and move faster than longer molecules through the pores of the gel and this process is called sieving. The gel might be used to look at the DNA in order to quantify it or to isolate a particular band. The DNA can be visualized in the gel by the addition of Ethidium bromide through gel documentation unit.

Agarose is a polysaccharide obtained from the red algae *Porphyra umbilicalis*. Its systematic name is (1 4)-3, 6-anhydro-a-L-galactopyranosyl-(1 3)-β-D-galactopyran. Agarose makes an inert matrix. Most agarose gels are made between 0.7% and 2% of agarose. A 0.7% gel will show good separation for large DNA fragments (5-10kb) and a 2% gel will show good resolution for small fragments with size range of 0.2-1kb. Low percentage gels are very weak (Note: it may break when you lift them) but high percentage gels are usually brittle and do not set evenly. The volume of agarose required for a mini gel preparation is around 30-50ml and for a larger gel, it is around 250ml. It is a powerful separation method frequently used to analyze DNA fragments generated by restriction enzymes, and it is a convenient analytical method for determining the size of DNA molecule in the range of 500 to 30,000 base pairs.

Working: The equipment requirements for conducting agarose gel electrophoresis start with three basic items:

1) Horizontal gel electrophoresis apparatus 2) Direct Current (D.C.) power source 3) Sample delivery instrument (automatic micropipette)

Dye electrophoresis experiments do not require additional equipment, although a visible light source (light box) will enhance visualization of the bands in the gel.

The horizontal electrophoresis apparatus is essentially a sophisticated rectangular-shaped "box" with electrodes at each end. All units found in research laboratories, contain platinum electrodes because of platinum's superior electrical conductivity and permanency. Because platinum electrodes are both expensive and fragile, care should be taken when handling electrophoresis equipment. The separation medium is a gel made from agarose, which is a non-toxic substance. The agarose is mixed with hydrocolloids which makes the gel clearer, more resilient and less prone to breakage. The gel is made by dissolving agarose powder in boiling buffer solution. The solution is then cooled to approximately 55°C and poured into a casting tray which serves as a platform. A well-former template (often called a comb) is placed across the end of gel solution casting tray to form wells. After the gel solidifies, the gel is submerged in a buffer-filled electrophoresis chamber which contains a positive electrode at one end, and a negative electrode at the other. Samples are prepared for electrophoresis by mixing them with components, such as glycerol or sucrose that will give the sample density. This makes the samples sink through the buffer and remain in the wells. These samples are delivered to the sample wells with a micropipette. A Direct Current (D.C.) power source is connected to the electrophoresis apparatus and electrical current is applied. Charged molecules in the sample enter the gel through the walls of the wells. Molecules having a net negative charge migrate towards the positive electrode (anode) while net positively charged molecules migrate towards the negative electrode (cathode). Within a range, the higher the applied voltage, the faster the samples migrate. The buffer serves as a conductor of electricity and to control the pH, which is important to the charge and stability of biological

molecules. Since DNA has a strong negative charge at neutral pH, it migrates through the gel towards the positive electrode during electrophoresis. If electrophoresis is conducted using dye samples, the migration of the various colored molecules can be visualized directly in the gel during electrophoresis and do not require staining. Because of the small size of the dye molecules, electrophoresis is fairly rapid. However, the small size of the dye molecules also makes them susceptible to diffusion out of the gel. Thus, the results of dye electrophoresis experiments must be viewed immediately when the separation is complete. Although DNA samples that are prepared for electrophoresis typically appear bluish-purple, the DNA itself does not have colour. The colour comes from a dye in a gel loading solution that is added at the end of typical DNA reactions, such as restriction enzyme digestion, or amplification by polymerase chain reaction. The gel loading solution stops there action. It also contains glycerol, which provides density to the sample so it will sink into the well during gel loading. The bluish-purple dye allows for visual tracking of sample migration during the electrophoresis. In general, most DNA samples follow behind the tracking dye during electrophoresis. Thus, it is important that electrophoresis is terminated before the tracking dye runs off the end of the gel.

The most commonly used stains for visualizing DNA contain either Ethidium bromide or methylene blue. Ethidium bromide is a mutagen and must be handled and disposed according to strict local and/or state guidelines. Visualization also requires a short wave ultraviolet light source (transilluminator). Stains containing methylene blue are considered safer than Ethidium bromide, but should still be handled and disposed with care.

Points to remember:

Preparing the Gel Bed

Close off the open ends of a clean and dry gel bed (casting tray) by using rubber dams or tape.

1. Using Rubber dams: Place a rubber dam on each end of the bed. Make sure the rubber dam fits firmly in contact with the sides and bottom of the bed.
2. Taping with labeling or masking tape: With 2 cm (3/4 inch) wide tape, extend the tape over the sides and bottom edge of the bed. Fold the extended edges of the tape back onto the sides and bottom. Press contact points firmly to form a good seal. Place a well-former template (comb) in the middle set of notches. Make sure the comb sits firmly and evenly across the bed.

Running the Gel

1. After the samples are loaded, carefully snap the cover down onto the electrode terminals.
2. Make sure that the negative and positive colour-coded indicators on the cover and apparatus chamber are properly oriented.
3. Insert the plug of the black wire into the black input of the power source (negative input). Insert the plug of the red wire into the red input of the power source (positive input).
4. Set the power source at the required voltage and conduct electrophoresis for the length of time determined by your instructor.
5. Check to see that current is flowing properly - you should see bubbles forming on the two platinum electrodes.
6. After approximately 10 minutes, you will begin to see separation of the colored dyes.
7. After the electrophoresis is completed, turn off the power, unplug the power source, disconnect the leads and remove the cover.
8. Document the gel results.

A variety of documentation methods can be used, including drawing a picture of the gel, taking a photograph, or scanning an image of the gel on a flatbed scanner.

Voltage Applied

The migration rate of the linear DNA fragments through agarose gel is proportional to the voltage applied to the system. As voltage increases, the speed of DNA also increases. But voltage should be limited because it heats and finally causes the gel to melt.

Ethidium Bromide (EtBr)

It is an intercalating agent which intercalates between nucleic acid bases and allows the convenient detection of DNA fragments in gel. When exposed to UV light, it will fluoresce with an orange colour. After the running of DNA through an EtBr-treated gel, any band containing more than ~20ng DNA becomes distinctly visible under UV light. EtBr is a known "mutagen", however, safer alternatives are available. It can be incorporated with agarose gels or DNA samples before loading, for visualization of the fragments. Binding of Ethidium bromide to DNA alters its mass and rigidity, and thereby its mobility.

Buffers

Several different buffers have been recommended for electrophoresis of DNA. The most commonly used buffers are Tris-acetate-EDTA (TAE) and Tris-borate-EDTA (TBE). The migration rate of DNA fragments in both of these buffers is somewhat different due to the differences in ionic strength. These buffers provide the ions for supporting conductivity.

Confirmation of DNA

DNA with different conformations that has not been cut with a restriction enzyme will migrate with different speeds. Nicked or open circular DNA will move slowly than linear and super coiled DNA (slowest to fastest: nicked or open circular, linear, or super coiled plasmid). Super helical circular, nicked circular and linear DNAs migrate gels at different rates through agarose gel. The relative nobilities of these three forms depend on the concentration, type of agarose used to make the gel, applied voltage, buffer, and the density of super helical twists.

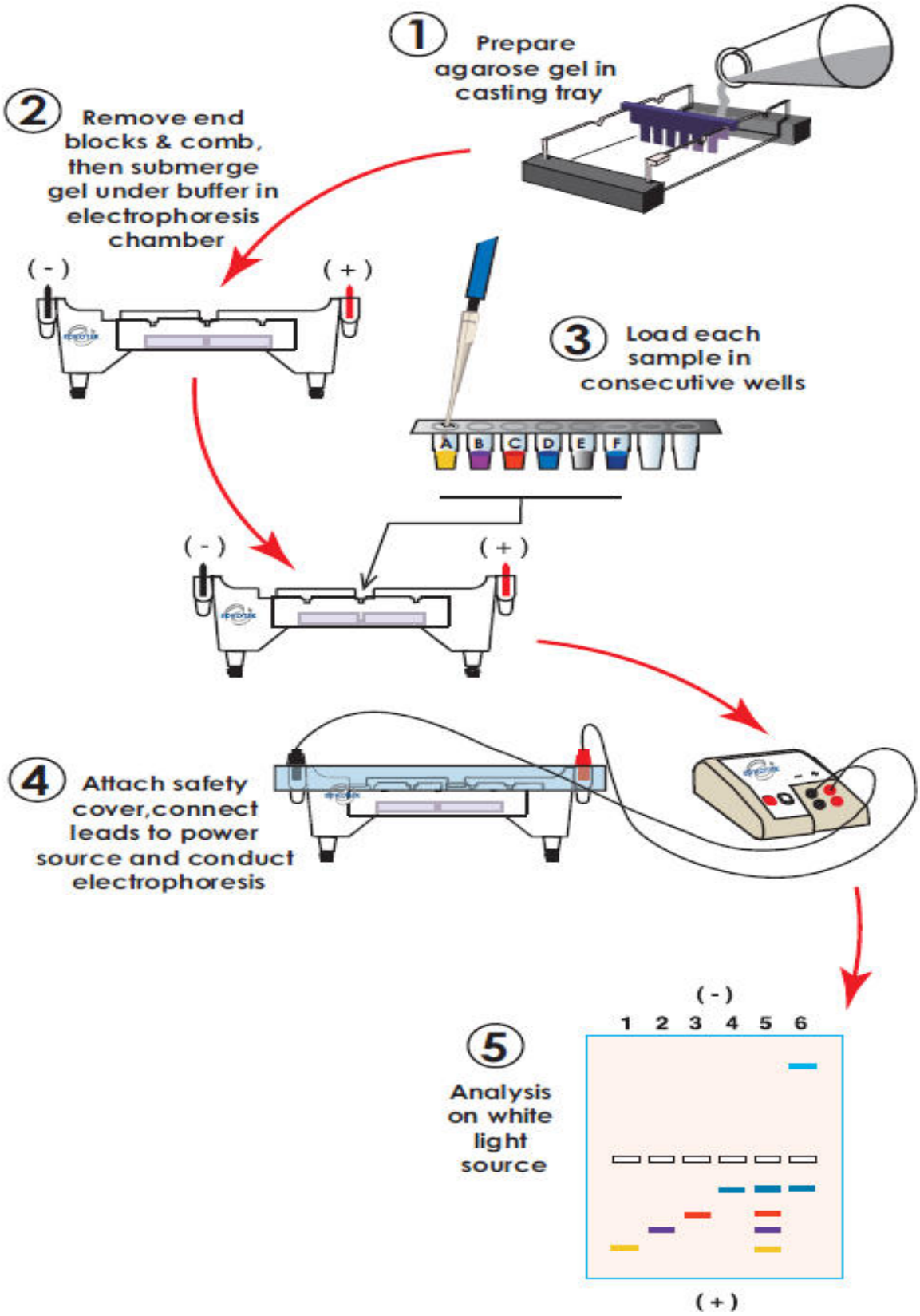


Fig. 15. Technical steps involved in Agarose Gel Electrophoresis

12. UV-Visible Spectrophotometer

General Description: To measure the chemical constituents of the sample and to get spectrometric analysis of the given samples.

Fig. 16. UV - Visible Spectrophotometer

Principle: If a beam of light of intensity falls on a cell containing the sample, the emergent radiation intensity I is less intense than the incident radiation, since a portion of it is absorbed by the sample. The absorption is different at different wavelengths and is characteristic of the sample. This characteristic quantity is called the absorbance and may be calculated from the Beer-Lambert principle. The Beer-Lambert law states that in a sample, each successive portion along the path of the incident radiation, containing an equal number of absorbing molecules absorbs an equal fraction of the radiation that traverses it.

A spectrophotometer measures the relative amounts of light energy passed through a substance that is absorbed or transmitted. We will use this instrument to determine how much light of (a) certain wavelength(s) is absorbed by (or transmitted through) a solution. Transmittance (T) is the ratio of transmitted light to incident light. Absorbance (A) = – log T. Absorbance is usually the most useful measure, because there is a linear relationship between absorbance and concentration of a substance. This relationship is shown by the Beer-Lambert law: A = ebc; Where e=extinction coefficient (a proportionality constant that depends on the absorbing species) b = path length of the cuvette. Most standard cuvettes have a 1 cm path and, thus, this can be ignored; c = concentration.

Working:

1. Turn on the spectrophotometer and allow 10 minutes for the instrument to warm up before use.
2. Adjust the wavelength to that specified for the procedure you are using. Be sure the cover is closed on the cuvette holder and use the left knob on the front panel to adjust the dark current so that the meter is reading zero transmittance. At this point, you are simply adjusting the internal electronics of the instrument to blank out any residual currents. This adjusts the lower limit of measurements. It establishes that no light is equivalent to 0 transmittance or infinite absorbance.
4. Insert a clean cuvette containing the blank into the holder. Be sure that the tube is clean, free of fingerprints, and that the painted line marker on the tube is aligned with the mark on the tube holder. Close the top of the tube holder. The blank for this exercise is the solution containing no dopachrome, but all other chemicals. The amount of solution placed in the cuvette is not important, but is usually about 5 ml. It should approximately reach the bottom of the logo printed on the side of the cuvette.
5. Adjust the meter to read 100% transmittance, using the right knob on the front of the instrument. This adjusts the instrument to read the upper limit of the measurements and establishes that your blank will produce a reading of 100% transmittance (zero absorbance).
6. Remove the blank from the instrument and recheck that your 0 transmittance value has not changed. If it does, wait a few minutes for the instrument to stabilize and read steps 1–5. Periodically throughout the work, check that the calibration of the instrument is stable by reinserting the blank and checking that the 0 and 100% T values are maintained.
7. To read a sample, simply insert a cuvette holding your test solution and close the cover. Read the transmittance value directly on the scale.
8. Record the percent transmittance of your solution, remove the test tube cuvette, and continue to read and record any other solutions you may have.

It is possible to read the absorbance directly, but with an analog meter (as opposed to a digital readout), absorbance estimations are less accurate and more difficult than reading transmittance. Absorbance can be easily calculated from the transmittance value. Be sure that you note which value you measure.

Absorption Spectrum

In the use of the instrument for determination of concentration (Beer-Lambert's Law), the wavelength was preset and left at a single value throughout the use of the instrument. This value is often given by the procedure being employed, but can be determined by an analysis of the absorption of a solution as the wavelength is varied.

A dual-beam spectrophotometer divides the light into two paths. One beam is used to pass through a blank, while the remaining beam passes through the sample. Thus, the machine can monitor the difference between the two as the wavelength is altered. It scans a blank and places the digitized information in its computer memory. It then rescans a sample and compares the information from the sample scan to the information obtained from the blank scan. Since the information is digitized (as opposed to an analog meter reading), manipulation of the data is possible. These instruments usually have direct ports for connection to personal computers, and often have built-in temperature controls as well. This latter option would allow measurement of changes in absorption due to temperature changes (known as hyperchromicity). These, in turn, can be used to monitor viscosity changes, which are related to the degree of molecular polymerization with the sample.

13. Water Bath

General Description: A water bath is an instrument used in the laboratory for carrying out biochemical, agglutination, inactivation and biomedical tests and industrial incubation procedures.

Fig. 17. Water Bath with Stirrer

Principle: It is a system for the control of temperature in which a vessel containing the material to be heated is set into or over one containing water and receiving the heat directly.

Working: It has a double walled, outer body made of MS sheet, powder coated, inner body made of stainless steel, and 304 quality sheet glass wool insulation, fitted with 30^{o}C to 110^{o}C thermostat. By means of these, heat is transferred to the medium (water or oil) until reaching the temperature selected with a control device (thermostat or similar). It is provided with concentric rings with a diameter of 75 mm suitable to work on 220V AC supply. It is accompanied with a digital display temperature controller. In general they use water, but some baths use oil.

Before using the water bath, verify that it is clean and that accessories needed are installed. The steps normally followed are:

1. Fill the water bath with fluid to keep the temperature constant (water or oil). Verify that once the containers to be heated are placed, the fluid level is between 4 and 5 cm from the top of the tank.
2. Install the control instruments needed, such as thermometers and circulators. Use additional mounts provided for this purpose. Verify the position of the thermometer's bulb or thermal probe to ensure that the readings are correct.
3. If water is used as the warming fluid, verify that it is clean. Some manufacturers recommend adding products which prevent the formation of fungus or algae.
4. Put the main switch Nº 1 in the ON position (the numbers identifying the controls herein correspond to those shown in the diagram). Some manufacturers have incorporated controls with microprocessors which initiate auto-verification routines once the ON switch is activated.
5. Select the operation temperature using the Menu Nº 2 button and the buttons for adjusting the parameters.

6. Select the cut-off temperature (in water baths with this control). This is a safety control which cuts off the supply of electricity if it exceeds the selected temperature. This is selected also by using the menu button and is controlled by the parameter adjustment buttons.
7. Avoid using the water bath with the substances indicated below:
Bleach, Liquids with high chlorine content, weak saline solutions such as sodium chloride, calcium chloride or chromium compounds, Strong concentrations of any acid, Strong concentrations of any salt, Weak concentrations of hydrochloric, hydrobromic, hydro iodic, sulphuric or chromic acids, Deionized water, as it causes corrosion and perforation in the stainless steel.

Safety

1. Avoid the use of the water bath in environments where there are flammable and combustible materials. The equipment has components (resistors generating very high temperatures) which could start an accidental fire or explosion.
2. Always connect the equipment to an electrical outlet with a ground pole to protect the user and the equipment from electrical discharges
3. Use the water bath exclusively with non-corrosive or non-flammable liquids.
4. When working with substances that generate vapours, place the water bath under a chemical hood or in a well-ventilated area.
5. Remember that liquids incubated in the water bath tank can produce burns if hands are inadvertently placed inside it.
6. Take into account that the water bath is designed for use with a liquid inside the tank. If the inside is dry, the temperature of the tank can become very high. Use the diffusing tray for placing the container inside of the filled tank of the water bath. This has been designed for distributing the temperature in a uniform way.
8. Avoid using the water bath if any of its controls is not working, e.g. the temperature or limit controls.

Cleaning

Frequency: Monthly

1. Turn off and disconnect the equipment. Wait until it cools to avoid the risk of burns and accidents.
2. Remove the fluid used for heating. If it is water, it can be poured through a siphon. If it is oil; collect into a container with an adequate capacity.
3. Remove the thermal diffusion grid located at the bottom of the tank.
4. Disassemble the circulator and clean to remove scale and potential algae present.
5. Clean the interior of the tank with a mild detergent. If there is any indication of corrosion, use substances for cleaning stainless steel. Rub lightly with synthetic sponges or equivalent. Avoid using steel wool to remove rust stains as these leave particles of steel which could accelerate corrosion.
6. Avoid bending or striking the temperature control capillary tube generally located at the bottom of the tank.
7. Clean the exterior and interior of the water bath with clean water.

14. Deep Freezer

Principle & Working: The basic principle behind a freezer is evaporation. When a liquid evaporates it causes the surrounding area to cool. Water can't be used in freezer though, because it evaporates at too high a temperature. But some liquids evaporate at very low temperatures. For example, Isobutane (becoming more common in domestic freezers) evaporates at very low temperatures. This ability to evaporate at very low temperatures means that it cools surfaces which are already very cold.

Fig. 18. Deep Freezer

Evaporation is affected by air pressure. The higher the air pressure, the less a liquid will evaporate.

1. The Compressor takes in the refrigerant (as gas); raise the air pressure which converts the refrigerant gas to liquid.
2. As the refrigerant liquid flows from the Compressor to the Expansion Valve the high air pressure stops it evaporating and instead it gives off heat and becomes cooler.
3. The refrigerant liquid flows through the expansion valve where the air pressure is much lower. This cause the refrigerant liquid to evaporate which cause the pipe to become very cold inside the freezer.
4. One key component of the freezer is the thermostat. The thermostat senses the temperature inside the freezer and when it drops below a certain temperature it turns off the motor so the flow of the refrigerant liquid stops. When the temperature rises above a certain level the thermostat turns on the motor and the refrigeration process restarts.

Refrigerant Liquids

Different freezers have different refrigerant liquids. Which liquid is used is important for two main reasons. Firstly different liquids are more or less efficient for use in a freezer. Less efficient liquids will use more electricity and therefore cost you more to run your freezer.

Secondly, some refrigerant liquids contribute significantly more than others to global warming and destruction of the ozone layer. Pre-1990 many freezers used CFC which caused significant environmental damage to the atmosphere. This was then replaced with MFC which does not destroy the ozone layer but still does contribute to global warming. Currently HC and Isobutane are used as refrigerant liquids in most domestic freezers. Both are good with Isobutane being the best.

15. Microscopes

General Description: Cells are small and in almost all situations a microscope is needed to observe them and their subcellular components. In fact the invention of the microscope led to the discovery and description of cells by Robert Hooke in 1655. The microscope is still an extremely important tool in biological research. The light microscope has a limited capability in regards to the size of a particle that can be examined.

Principle & Working:

15 a. Light Microscopy

The principal of light microscopy is to shine light through a specimen and examine it under magnification. The major optical parts of a microscope are the objective lens, the eyepiece, the condenser and the light source. The objective lens functions to magnify the object. The high degree of magnification of the objective lens results in a small focal length and the magnified image actually appears directly behind the objective. The eyepiece functions to deliver this image to the eye or camera. Eyepieces also magnify the image, but it is an empty magnification. In other words, the eyepiece enlarges the image but does not increase the ability to see fine details (i.e., the resolution). The condenser functions to focus the light source on the specimen. The condenser also eliminates stray light and provides a uniform illumination. An iris diaphragm which controls the amount of light reaching the specimen is also associated with the condenser lens. In addition, the light intensity can also be controlled by adjusting the voltage applied to the lamp on some microscopes.

Before using a microscope it is also important to check that all of the optical components are centered on an optical axis so that the best image and resolution are obtained. Aligning the optical components is usually simple and needs to be done periodically. The specimen is then placed on the stage and the objective lens is focused. The quality of the image produced is highly dependent on the illumination. The position of the condenser lens is adjusted so that the light is focused on the specimen and the intensity of the illumination is adjusted. On better microscopes the illumination can be controlled by both adjusting the diameter of the iris and by adjusting the voltage applied to the lamp. The amount of illumination is important for controlling resolution vs. the contrast and the depth of field. Resolution and contrast are antagonistic in that improving one result in a loss of the other.

15 b. Stereoscopic Zoom Microscope

A stereo microscope is a tool for magnifying a three-dimensional object in three dimensions; Magnification, zoom range and object field. The total magnification of stereo microscopes is the combined magnification of the magnification changer, the objective and the eyepieces.

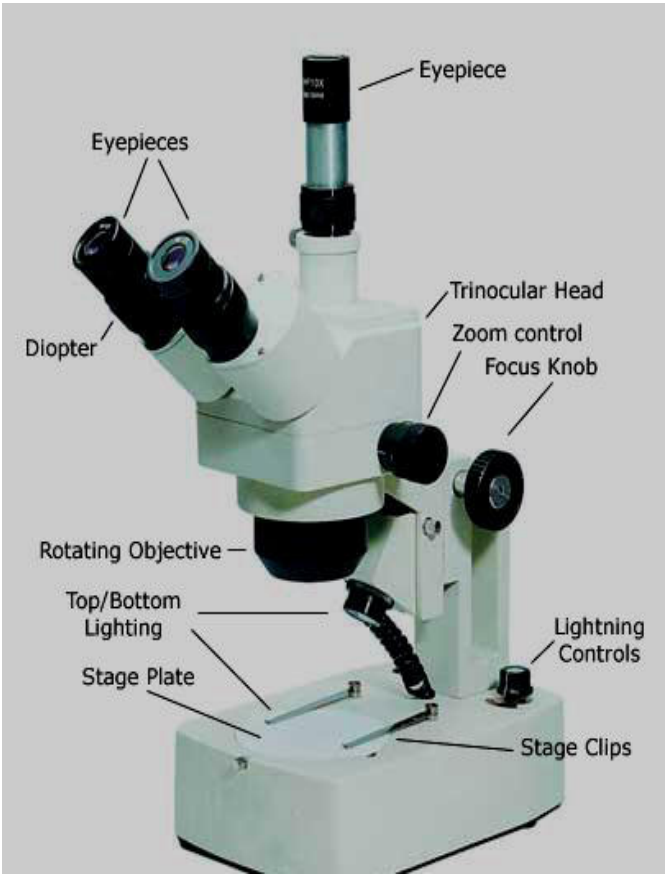


Fig. 19. Stereoscopic Microscope

- The magnification changer or zoom body: Like a magnifying glass, the magnification changer consists of optical lenses that can be used to change the magnification of the instrument. Changing the position of the magnification changer alters the degree to which the image is magnified. The degree to which the image is magnified is called the magnification factor. Modern stereo microscopes are able to provide up to 16x magnification (zoom body only) with a 20.5:1 zoom range and feature motorization or encoding to allow reliable measurement. The eyepieces: To find out the magnification of the object he or she is observing in the eyepieces, the user has to multiply the magnification factors of the magnification changer and the eyepieces.
- The objectives: Most stereo microscopes have dual objectives designed for viewing without cover slips. They are designed to view objects at relatively low magnifications, typically 10x - 40x. For most stereo microscopes, working distance (the distance from the bottom of an objective to the in-focus area of an object) and depth of field are relatively large. Resolution and working distance typically have an inverse relationship. Stereo microscopes provide microscopic views of the world without the need for complex object preparation. Because of their large field of view they can give us "in context" views of objects that would otherwise be impossible to obtain.
- An object's image is not reversed by a stereo microscope. That is, moving an object to the left moves its image to the left, and moving an object downward moves its image downward. Thus, "abc" seen under a stereo microscope appears as "abc".

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6

Tabulations

Units & measurements

Length

1 micron= 1µ= 1µ m=$1x10^{-6}$m=$1x10^{3}$nm=$1x10^{4}$Å
1Å= 0.1 nm= $1x10^{-4}$µm= $1x10^{-10}$m
1 nm= 10 Å= 1x 10^{-3}µm= 1x 10^{-9}m

Volume

1ml = 1000µl
1ml = $1cm^{3}$
1 gallon = 3.8l
1 ounce = 29.6ml

Weight

1kg = 1000g
1mg = 0.001g
1µg = 0.000001g

Conversions

1ppm = parts per million = mg/l= µg/ml
1g/1000ml = 1000 ppm

Dilution Formula

Desired Concentration of auxin and /or cytokinin are added from the stock solution according to the formula-

Desired concentration/Stock Concentration=Amount (ml) of stock solution to be taken for one litre medium.

If the quantity of the medium is less than one litre, then hormones are added using another formula-

(Required Concentration X Volume of Medium)/Stock Concentration X 1000 = Amount (ml) of stock solution to be added.

To know the unknown concentration we have the formula:

$V_1C_1=V_2C_2$

Where, V_1= Initial Volume; C_1= Initial Concentration; V_2= Final Volume; C_2= Final Concentration

Percent Solution: One percent solution of a substance contains one gram of the substance in 100ml of the solvent. If v/v is given it is 1ml in 100ml of solution.

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7

Preparation of Solutions

Preparation of Solutions

(a) Calculation of Molar, %, and "X" Solutions

(*i*) A molar solution is one in which 1 litre of solution contains the number of grams equal to its molecular weight.

Example. To make up 100 ml of a 5M NaCl solution = 58.456 (mw of NaCl) g × 5 moles × 0.1 liter = 29.29 g in 100 ml sol mole liter.

(*ii*) *Percent solutions*

Percentage (*w*/*v*) = weight (g) in 100 ml of solution

Percentage (*v*/*v*) = volume (ml) in 100 ml of solution.

Example. To make a 0.7% solution of agarose in TBE buffer, weigh 0.7g of agarose and bring up the volume to 100 ml with the TBE buffer.

(*iii*) *"X" solutions.* Many enzyme buffers are prepared as concentrated solutions,

e.g., 5 X or 10 X (5 or 10 times the concentration of the working solution), and are then diluted so that the final concentration of the buffer in the reaction is 1 X.

Example. To set up a restriction digestion in 25 ml, one would add 2.5 ml of a 10 X buffer, the other reaction components, and water for a final volume of 25 ml.

(b)Standard NaOH Solution (0.1M/0.1N)

Take 0.4g NaOH and dissolve gradually with 100ml sterile distilled water.

(c) Standard HCl Solution (0.1M/0.1N)

Dilute 9 ml of pure concentrated HCl to 1.0 l with distilled water in volumetric flask. Invert several times and transfer to a clean, dry bottle.

(c) Steps in Solution Preparation

(*i*) Refer to the laboratory manual for any specific instructions on preparation of the particular solution and the bottle label for any specific precautions in handling the chemical.

(*ii*) Weigh out the desired amount of chemical(s). Use an analytical balance if the amount is less than 0.1 g.

(*iii*) Pour the chemical(s) in an appropriate size beaker with a stir bar.

(*iv*) Add less than the required amount of water. Prepare all solutions with double-distilled water.

(*v*) When the chemical is dissolved, transfer to a graduated cylinder and add the required amount of distilled water to achieve the final volume.

An exception is when preparing solutions containing agar or agarose. Weigh the agar or agarose directly in the final vessel.

(*vi*) If the solution needs to be at a specific pH, check the pH meter with fresh buffer solutions and follow the instructions for using a pH meter.

(*vii*) Autoclave, if possible, at 121°C for 20 minutes. Some solutions cannot be autoclaved; for example, SDS. These should be filter-sterilized through a 0.22 mm filter. Media for bacterial cultures must be autoclaved the same day it is prepared, preferably within an hour or two. Store at room temperature and check for contamination prior to use by holding the bottle at eye level and gently swirling it.

Table 2. Hormones used in a biotechnology laboratory

Hormones	Solvents Used	Concentration	Storage at 0°C (days)
Auxins (a) α- Naphthalene Acetic Acid (NAA)	Absolute Ethanol/ 1N NaOH	0.5mg/ml	7
(b) 3- Indole Butyric Acid (IBA)	Absolute Ethanol/ 1N NaOH	0.5mg/ml	7
(c) Indole Acetic Acid (IAA)	Absolute Ethanol/ 1N NaOH	0.5mg/ml	7
(d) 2,4-dichlorophenoxy acetic acid	Absolute Ethanol/ 1N NaOH	0.5mg/ml	7
Cytokinins (a) 6- benzyl amino purine(BAP)	1N HCl	0.5mg/ml	7
(b) Kinetin (6- furfuryl amino purine)	1N HCl	0.5mg/ml	7

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8

Basics of Plant Tissue Culture

Plant tissue culture is the science of growing plant cells, tissues or organs isolated from healthy, superior quality mother plant to artificial medium.

Culture of Organized Structures

Organ culture is used as a general term for those types of culture in which an organized form of growth can be continuously maintained. It includes the aseptic isolation from whole plants of such definite structures as leaf primordia, immature flowers and fruits, and their growth *in vitro*. The most important kinds of organ culture are:

1. Meristem cultures: They are grown as very small excised shoot apices, each consisting of the apical meristematic dome with or without one or two leaf primordia. The shoot apex is typically grown to give one single shoot.
2. Shoot tip or shoot cultures: It starts from excised shoot tips or buds, larger than the shoot apices employed to establish meristem cultures, having several leaf primordia. These shoot apices are usually cultured in such a way that each produce multiple shoots.
3. Node cultures: The separate lateral buds, each carried on a small piece of stem tissue or stem section carrying either single or multiple nodes can be cultured. Each bud is grown to provide a single shoot.
4. Isolated root cultures: The growth of roots, unconnected to shoots through which a branched root system may be obtained.
5. Embryo cultures: They were fertilized or unfertilized zygotic (seed) embryos which are dissected out of developing seeds or fruits and cultured *in vitro* until they have grown into seedlings. Embryo culture is quite distinct from somatic embryogenesis.

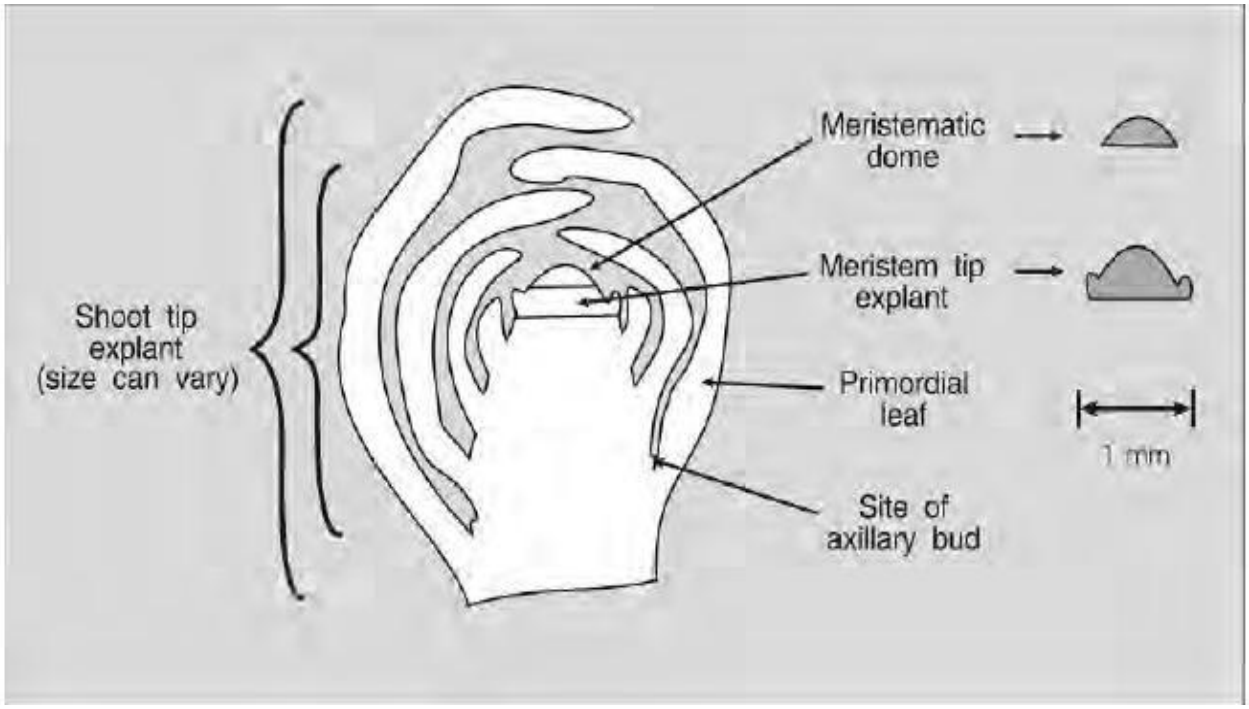

Fig. 20. A diagrammatic section through a bud showing location and approximate relative sizes of a meristematic dome, the meristem tip and shoot tip explants.

Culture of Unorganized Tissues

In practice the following kinds of cultures are most generally recognized:

1. Callus (or tissue) cultures: The growth and maintenance of largely unorganized cell masses, which arise from the uncoordinated and disorganized growth of small plant organs, pieces of plant tissue or previously cultured cells.
2. Suspension (or cell) cultures: Population of plant cells and small cell clumps, dispersed in an agitated liquid medium.
3. Protoplast cultures: The culture of plant cells that have been isolated without a cell wall.
4. Anther cultures: The culture of complete anthers containing immature pollen microspores. The objective is usually to obtain haploid plants by the formation of somatic embryos directly from the pollen, or sometimes by organogenesis *via* callus.
5. Pollen cultures: They are those initiated from pollen that has been removed from anthers.

Initiating Tissue Cultures

Explants: Tissue culture starts from pieces of whole plants. The small organs or pieces of tissue that are used are called explants. The part of the plant (mother plant) from which explants are obtained depends on the kind of culture to be initiated, the purpose of the proposed culture and the plant species to be used.

Plants growing in the external environment are invariably contaminated with micro-organisms and pests. These contaminants are mainly confined to the outer surfaces of the plant, although, some microbes and viruses may be systemic within the tissues. Since they start from small explants and to be grown on nutritive media that are also favorable for the growth of microorganisms they must be free from microbial contaminants when they are first placed on a nutrient medium. This usually involves growing mother plants in ways that will minimize infection, treating the explant material with disinfecting chemicals to kill superficial microbes and sterilize the tools used for dissection and the vessels and media in which cultures are grown.

Isolation and incubation: The work of isolating and transferring cultured planting material is usually performed in special rooms or Laminar air flow cabinets from which microorganisms can be excluded. Cabinets used for isolation are placed in a special inoculation room reserved for the purpose. Cultures, once initiated are placed in incubators or growth rooms where lighting, temperature and humidity are controlled. The rate of growth of a culture will depend on the temperature regime adopted.

Fig. 21. Tissue Culturist Inoculating in Laminar Air Flow Chamber

The cultural environment: Plant tissue cultures are commenced by placing one or more explants into a pre-sterilized container of sterile nutrient medium. Initiate several cultures at the same time, each being started from an identical organ or piece of tissue. Explants taken from mother plants at different times of the year may not give reproducible results in tissue culture. This may be due to variation in the level of external contaminants or because of seasonal changes in endogenous (internal) growth regulator levels in the mother plant.

Pattern of Growth and Differentiation: A typical unorganized plant callus, initiated from a new explant or a piece of a previously-established culture has three stages of development, namely: the induction of cell division; a period of active cell division during which differentiated cells lose any specialized features they may have acquired and become dedifferentiated; a period when cell division slows down or ceases and when, within the callus, there is increasing cellular differentiation.

Fig. 22. Tissue Culture Stand with Cultures in Incubation Room

Subculturing

Once a particular kind of organized or unorganized growth has been started *in vitro*, it will usually continue if callus cultures, suspension cultures, or cultures of indeterminate organs are undergone divisions. Subculturing often becomes imperative to increase the volume of a culture; or to increase the number of organs (e.g. shoots or somatic embryos) for micropropagation. The period from the initiation of a culture or a subculture to the time of its transfer is sometimes called a passage. The first passage is that in which the original explant or inoculum is introduced. Subculture is therefore more conveniently carried out during the stationary phase when cell aggregation is least pronounced. Rapid rates of plant propagation depend on the ability to subculture shoots from proliferating shoot or node cultures. A further reason for transfer, or subculture, is that the growth of plant material in a closed vessel eventually leads to the accumulation of toxic metabolites and the exhaustion of the medium, or to its drying out. Thus to maintain the culture, all or part of it must be transferred onto fresh medium.

Callus subcultures are usually initiated by moving a fragment of the initial callus (an inoculum) to fresh medium in another vessel. Shoot cultures are subcultured by segmenting individual shoots or shoot clusters. The interval between subcultures depends on the rate at which a culture has grown: at 25°C, subculturing is typically required every 4-6 weeks. In the early stages of callus growth it may be convenient to transfer the whole piece of tissue to fresh medium, but a more established culture will need to be divided and only small selected portions used as inocula.

Cultures which are obviously infected with micro-organisms should not be used for subculturing and should be autoclaved before disposal.

Rooting

Single shoots or shoot clusters moved to a different medium for rooting *in vitro* before being transferred as plantlets to the external environment. For this auxins are supplied in specific quantities or sometimes a hormone free medium is suitable for rooting. An alternative strategy for some plants is to root the plant material *ex vitro*. Treatments need to be varied according to the type of growth; the nature of the shoot proliferation produced during subculture; and the plant habit required by the customer.

Table 3. Stages of cultures in micropropagation

	Stages of culture		
Methods of Micropropagation	I. Initiating a culture Growth of excited tissues/organs *invitro* free from algae, bacteria, fungi and other contaminants	II. Increasing propagules Inducing the cultures to produce number of shoots or somatic embryos.	III. Preparation for soil transfer Separating and preparing propagules to have a high rate of survival as individual plants in the external environment.
Shoot Cultures	Transfer of disinfected shoot tips or lateral buds to solid or liquid media and the commencement of shoot growth to ca. 10mm.	Induce multiple axillary shoot formation and growth of the shoots to a sufficient size for separation, either as new stage II explants or for passage to III.	Elongation of buds formed at Stage II to uniform shoots. Rooting the shoots in vitro or outside the culture vessel.
Shoots from floral Meristems	Aseptic isolation of pieces of compound floral meristems.	Inducing the many meristems to produce vegetative shoots, then as shoot tip culture.	As for shoot tip cultures.
Multiple shoots from seeds	Aseptic germination of seeds on a high cytokinins medium.	Inducing multiple shoot proliferation. Shoot subculture	As for shoot tip cultures.
Meristem culture	Transfer of very small shoot tips (length 0.2-0.5mm) to culture. Longer shoot tips (1-2mm) can be used as explants if obtained from heat treated plants.	Growth of shoots to ca. 10mm, then as shoot tip culture, or as shoot multiplication omitted and shoots transferred to Stage III.	As for shoot tip cultures.
Node culture	As for shoot tip culture but shoots grown longer to show clear internodes.	Propagation by inducing the axillary bud at each node to grow into a single shoot. Subculturing can be repeated indefinitely.	As for shoot tip cultures.
Direct shoot regeneration from explants	Establishing suitable explants of mother plant tissue (e.g. leaf or stem segments) in culture without contamination.	The induction of shoots directly on the explant with no prior formation of callus. Shoots so formed can usually be divided and used as explants for new Stage II subcultures or shoot tip culture.	As for shoot tip cultures
Direct embryogenesis	Establishing suitable embryogenic tissue explants or previously-formed somatic embryos.	The direct induction of somatic embryos on the explants without prior formation of callus.	Growth of the embryos into plantlets which can be transferred to the outside environment.

Indirect shoot regeneration from morphogenic callus	Initiation and isolation of callus with superficial shoot meristems.	Repeated subculture of small callus pieces followed by transfer to a shoot-inducing medium. The growth of shoots ca. 10mm in length.	Individual shoots are grown and rooted.
Indirect embryogenesis from embryogenic callus or suspension cultures	Initiation and isolation of callus with the capacity to form somatic embryos, OR obtaining embryogenic suspension cultures from embryogenic callus or by de novo induction.	Subculture of the embryogenic callus or suspension culture followed by transfer to a medium favouring embryo development.	Growth of the somatic embryos into plantlets which can be transferred to the outside environment.
Storage organ formation	Isolation and culture of tissue/organs capable of forming storage organs.	Inducing the formation storage organs and sometimes dividing them to start new Stage II cultures.	Growing shoots/plantlets obtained from storage organs for transfer to soil: OR growing the storage organs themselves to a size suitable for soil planting.

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9

Plant Tissue Culture Medium

Culture Medium

The planting material will grow *in vitro* only when they are aseptically inoculated onto sterile medium. A medium is a complete mixture of nutrients and growth regulators. Here growth regulators are frequently altered according to the variety of plant, or at different stages of culture, while the basic medium can stay unchanged. Plant material can be cultured either in a liquid medium or on a medium that has been partially solidified with a gelling agent.

The medium consisted of salts (Calcium chloride) and vitamins defined by Murashige and Skoog (1962), which is widely used medium for tissue culture. Commercial grade Sucrose (3%) was used as sole carbon source separately. The medium is gelled with 0.65% agar. Phytohormones like auxins, cytokinins are used. The pH of the medium is adjusted to 5.7 (for pineapple it is 5.6) before autoclaving at a pressure 15 lb and temperature raised to 121°C.

Media Components

One of the most important factors governing the growth and morphogenesis of plant tissues in culture is the composition of the culture medium. The basic nutrient requirements of cultured plant cells are very similar to those of whole plants. Plant tissue culture media are generally made up of some or all of the following components.

Table 4. Composition of Murashige & Skoog Medium (1962)

Stock solution Code No.	Constituent chemicals	Quantity (g/l)	Stock concentration
1	$NH_4(SO4)_2$	82.5	50X
	KNO_3	95.0	
	$MgSO_4.7H_2O$	18.5	
	KH_2PO_4	08.5	
		Dissolved in 1000 ml sterile distilled water. Take 20 ml for 1l medium.	
2	$CaCl_2.2H_2O$	22	100X
2)		Dissolved in500 ml sterile distilled water. Take 10 ml for 1l medium.	
3	Na_2 EDTA	1.865	100X
	$FeSO_4.7H_2O$	1.399	100X
		Dissolve Na_2 EDTA completely and then add $FeSO_4.7H_2O$. Make up to 500ml using sterile distilled water. For 1l medium take 10ml.	
4	$MnSO_4.4H_2O$	22.3	2000X
	$ZnSO_4.7H_2O$	8.6	2000X
	$CuSO_4.5H_2O$	0.025	2000X
	$CoCl_2.6H_2O$	0.025	2000X
	KI	0.83	2000X

	$Na_2MoO_4.2H_2O$	0.25	2000X
	H_3BO_3	6.2	2000X
		Dissolve sulphates separately one by one using sterile distilled water. Then add Boric acid. Make up the volume to 500ml. Take 0.5ml for 1l medium.	
5	Glycine	0.2	400X
	Nicotinic acid	0.05	400X
	Pyridoxine HCl	0.05	400X
	Thiamine HCl	0.01	400X
	Myo- inositol	10	400X
		Dissolved each ingredient and make up the volume to 250ml using sterile distilled water. Take 2.5ml for 1l medium.	

Note: All stock solutions should be stored in refrigerator. They should be used until 3 months. Always prepare stock with sterile distilled water and label stock solution with dates. Any stock solution containing cloudiness or has bacterial or fungal contamination should be discarded. The pH of the medium should be adjusted before adding agar and autoclaving.

Plant growth regulators (PGRs)

Plant growth regulators are compounds, which, at very low concentration, are capable of modifying growth or plant morphogenesis

a. Auxins

- concerned with cell division
- cell elongation, formation of meristems
- maintenance of apical dominance and mediation of tropisms
- They are used in plant tissue culture and form an integral part of nutrient medium.

b. Cytokinins

- stimulate protein synthesis
- participate in cell cycle control
- stimulate cell division, induce shoot formation
- axillary shoot proliferation
- inhibit root formation and control morphogenesis

Other addenda ingredients

a. Activated charcoal

Activated charcoal or activated carbon is an amorphous form of carbon.

The effect of activated charcoal attributed to the following factors:

- absorption of inhibitory compounds
- absorption of growth regulators from the culture medium
- darkening of the medium
- prevent unwanted callus growth
- Promote morphogenesis, particularly embryogenesis

  It also promotes root formation (because of the ability to exclude light from the medium).

b. Agar

A gelatinous substance derived from red algae primarily from the genera *Gelidium* and *Gracilaria*, or seaweed (*Sphaerococcus euchema*). Cultures grown on solid media are kept static. They require only simple containers of glass or plastic, which occupy little space. Only the lower surface of the explant, organ or tissue is in contact with the medium. This means that as growth proceeds there may be gradients in nutrients, growth factors and the waste products of metabolism, between the medium and the tissues. Gaseous diffusion into and out of the cells at the base of the organ or tissue may be restricted by the surrounding medium.

It has several advantages over other gelling agents.

- When agar is mixed with water, it formed a gel that melted at approximately 60°-100°C and solidify at approximately 45°C; thus, agar gels are stable at all feasible incubation temperatures.
- Additionally, agar gels do not react with media constituents and are not digested by plant enzymes.
- The firmness of an agar gel was controlled by the concentration and brand of agar used in the culture medium and the pH of the medium.
- The agar concentrations commonly used in plant cell culture media range between 0.5 and 1.0%; these concentrations gave a firm gel at the pH's typical of plant cell culture media.

pH

- Relative acidity and alkalinity is assessed by the pH.
- Nutrient medium pH ranges from 5.5-6.0 for suitable *in vitro* growth of explants.
- It has effect in chemical reactions and also affects gelling efficiency of agar.
- The pH higher than 7.0 and lower than 4.5 generally stop growth and development.
- The pH before and after autoclaving is different. It generally falls by 0.3-0.5 units after autoclaving.

It is known that a starting pH of 5.8 could often fall to 5.5 or even lower during growth.

Liquid media

Liquid media are essential for suspension cultures, and are preferred for critical experiments on the nutrition, growth and cell differentiation in callus tissues. They are also used in some micropropagation work. Very small organs (e.g. anthers) are often floated on the top of liquid medium and plant cells or protoplasts can be cultured in very shallow layers of static liquid, provided there is sufficient gaseous diffusion. Larger organs such as shoots (e.g. Proliferating shoots of shoot cultures) can also be grown satisfactorily in a shallow layer of non-agitated liquid where part of the organ protrudes above the surface. However, some method of support is necessary for small organs or small pieces of tissue, which would otherwise sink below the surface of a static liquid medium, or they will die for lack of aeration. The purposes served by agitation include: the provision of increased aeration, the reduction of plant polarity, the uniform distribution of nutrients and the dilution of toxic explant exudates.

Problems of Cell Culture Establishment

1. Phenolic oxidation

Some plants, particularly tropical species, contain high concentrations of phenolic substances that are oxidized when cells are wounded or senescent. Isolated tissue then becomes brown or black and fails to grow.

2. Minimum inoculation density

Certain essential substances can pass out of plant cells by diffusion. Substances known to be released into the medium by this means include alkaloids, amino acids, enzymes, growth substances and vitamins. The loss is of no consequence when there is a large cluster of cells growing in close proximity or where the ratio of plant material to medium is high. However, when cells are inoculated onto an ordinary growth medium at a low population density, the concentration of essential substances in the cells and in the medium can become inadequate for the survival of the culture. For successful culture initiation, there is thus a minimum size of explant or quantity of separated cells or protoplasts per unit culture volume. Inoculation density also affects the initial rate of growth *in vitro*. Large explants generally survive more frequently and grow more rapidly at the outset than very small ones. In practice, minimum inoculation density varies according to the genotype of plant being cultured and the cultural conditions. For commencing suspension cultures it is commonly about 1-1.5 x 104 cells/ml.

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10

Pineapple Tissue Culture

Micro propagation of pineapple is done to produce large number of virus free planting material and to satisfy the large need of planting materials for elite pineapple varieties.
The varieties we focus for tissue culture are MD-2, Kew, Amrita, Mauritius and Baby Pineapple.

It involves the following stages.

1. **Initiation**
   a. Selection of explant
   b. Sterilization treatments for explant
   c. Fresh inoculation of explant
   d. Media change using fresh media
2. **Multiplication**
   a. Subculture for callus formation
   b. Shoot initiation
3. **Rooting**
   a. Healthy shoots transfer to rooting medium
   b. Cultures for sale
4. **Hardening**
   a. Treatments before planting out
   b. Mist chamber
   c. Green House
   d. Field Planting

1. Initiation

The types of explants used for tissue culture are suckers, slips and crown of the pineapple plant. It should satisfy all the criteria mentioned for the quality of explant.

Preparation of explants and fresh inoculation

- Roots and leaf sheaths are removed from the sucker, and basal portion of the sucker is cut and trimmed to a size of 12x12x15 mm
- Keep the explants under running tap water for 30', then soak in cleansol (detergent) and shake in orbital shaker for 30 minutes and are shaken continuously
- Wash with distilled water to remove the detergent particles
- Treat with fungicide [SAAF (0.05%)+INDOFIL(0.1%)+BAVISTIN(0.1%)] for 30' in orbital shaker followed by distilled water wash
- They are then transferred to laminar air flow chamber for further sterilization process
- Inside the laminar air flow chamber, the explants are stirred with 70% ethanol for 2'
- Wash with sterile water
- After that the explants are stirred with 0.1% $HgCl_2$ for 5'
- Three rinsing of 5' each with sterile distilled water
- The explants are trimmed to a final size of 1x1x1cm, [sometimes the explant may be dipped in Gentamycin (2ml/l)] in sterile conditions inoculated to the media
- Incubate at 25+/- 2 ^{o}C for 21 days

The inoculated explants will show bulging and new bud formation within 7 days. After 21 days they are transferred to bud proliferation media for increase in number of buds. Again after 21 days the cultures are transferred to multiplication media.

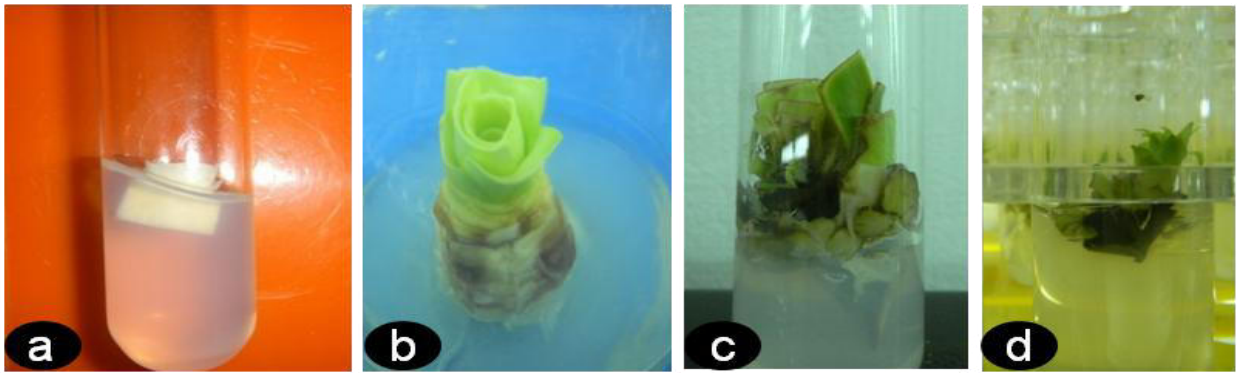


Fig. 23. Kew fresh inoculation: (a) 0th day (b) after 7 days(c) after 14 days(d) after 21 days

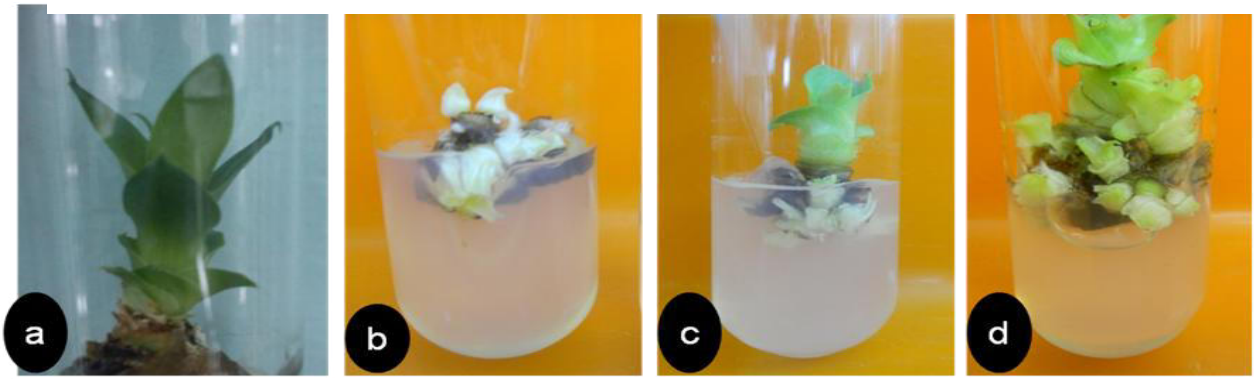


Fig. 24. Kew Bud proliferation: (a) 0th day (b) after 7 days (c) after 14 days (d) after 21 days

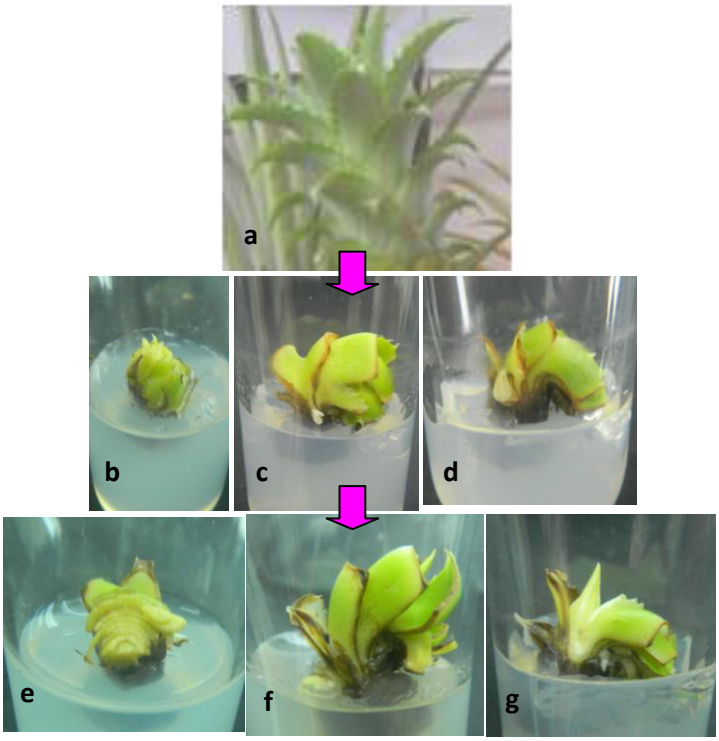


Fig. 25. Fresh Inoculation of Baby Pineapple (BP). a. Crown explant (b-d) Periodical Response of BP during Fresh Inoculation and Media Change (e-g)

4. **Multiplication**

This step consists of separating buds, culturing them up to form callus. When they reach optimum growth, transfer them to fresh culture medium for multiplication and again going through the same cycle of activities for another subculture. This step is repeated for eight to ten cycles.

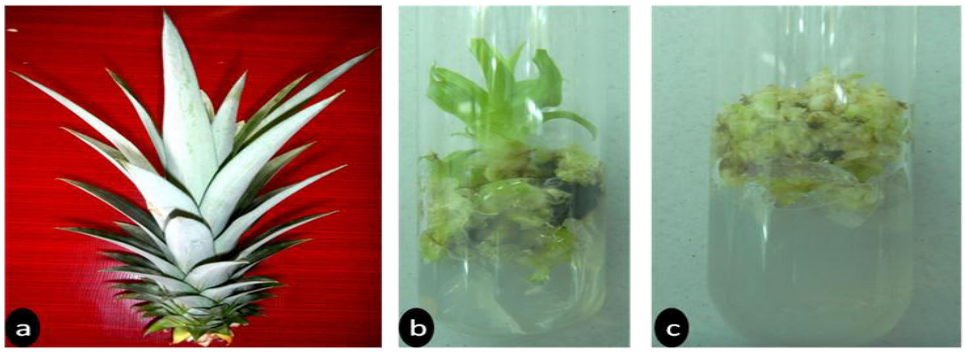


Fig. 26. MD-2 Callus initiation via crown: (a) Crown (b) 4th subculture after 21 days (c) 5th subculture after 21 days

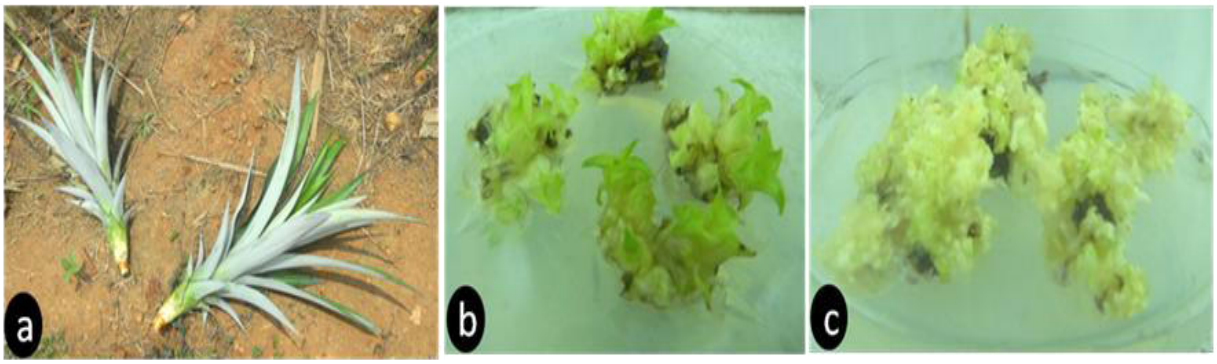


Fig . 27. MD-2 Callus initiation via sucker: (a) Sucker (b) 4th subculture after 21 days (c) 5th subculture after 21 days

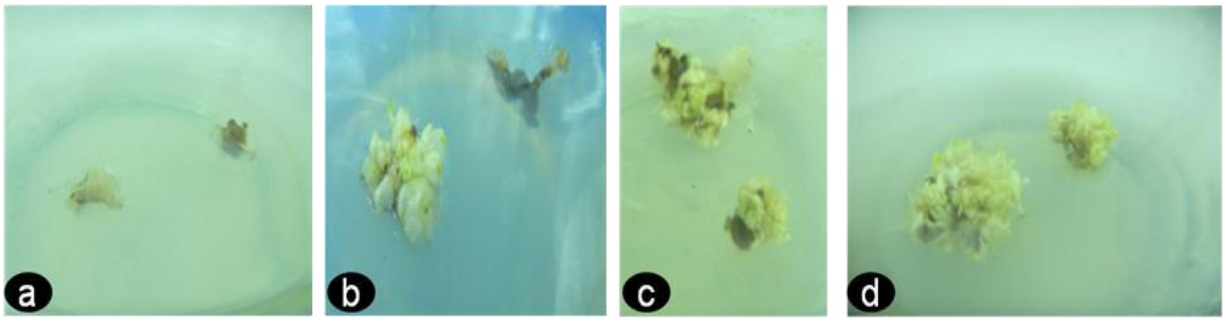


Fig. 28. Kew Callus Initiation: (a) 0th day (b) after 7 days (c) after 14 days (d) after 21 days

Multiplied calli are transferred to shoot initiation media for the development of shoots. It takes two months of duration for the proper development of shoots in the media.

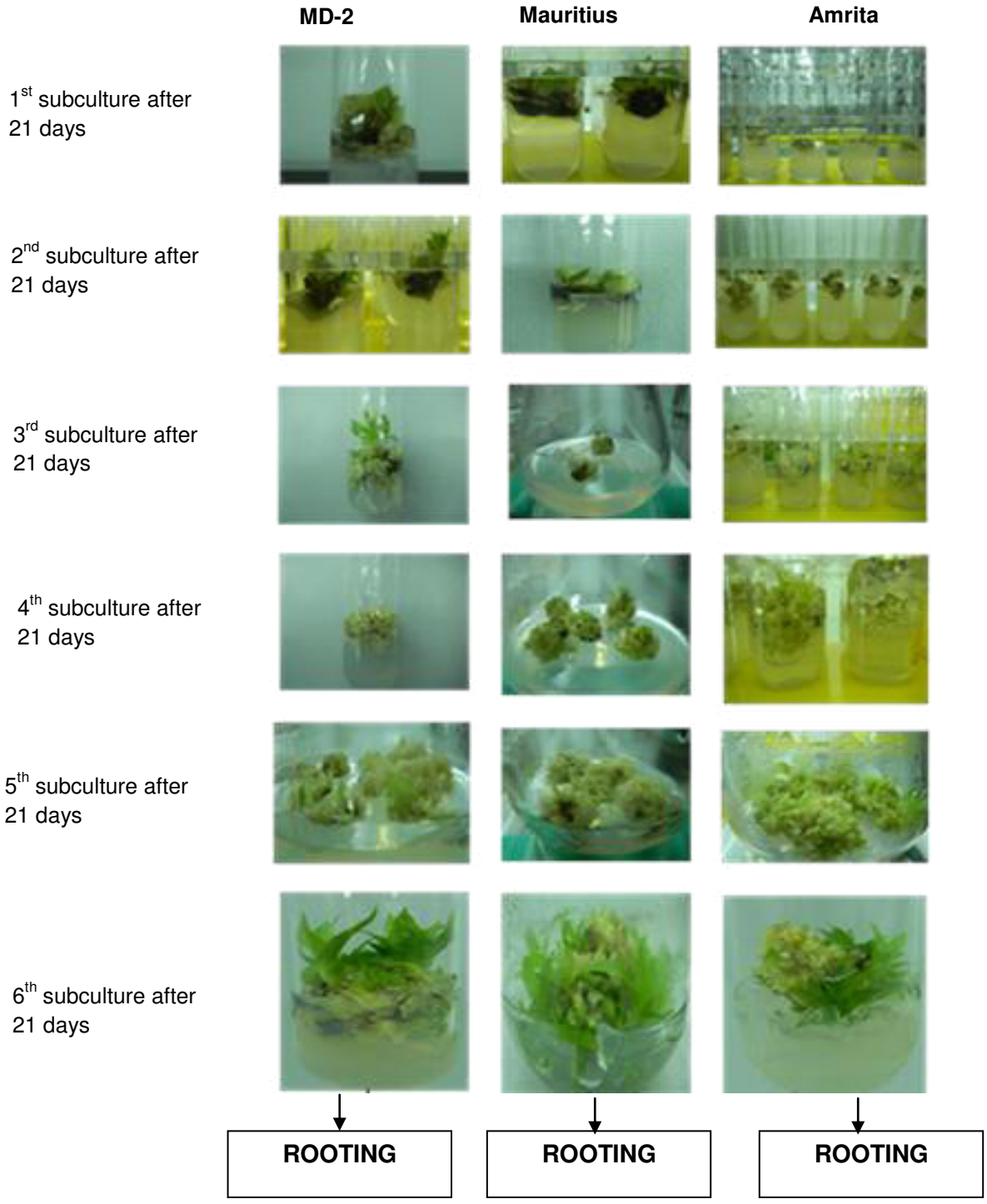


Fig. 29. Sequential sub culturing and multiplication of MD-2, Mauritius and Amrita pineapple

5. Rooting

After two months of time, the developed shoots are placed in to the root initiation media for the generation of roots. The development of root in pineapple is very slow and time consuming and it takes normally about 3 months.

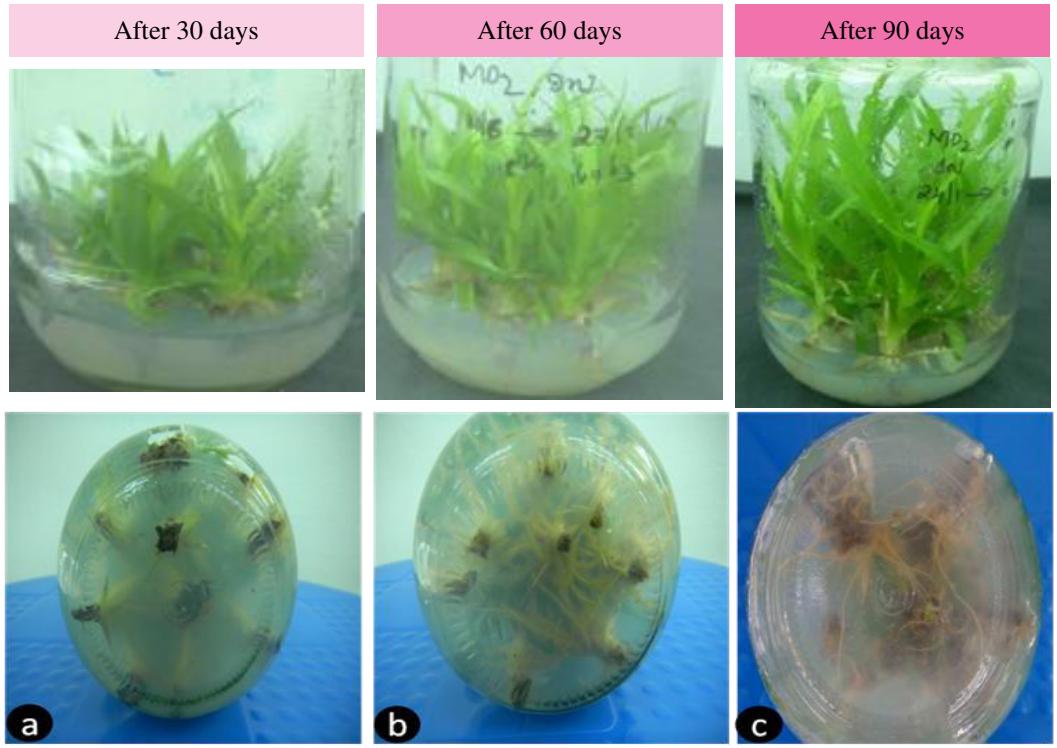


Fig. 30. Periodical Response of MD-2 Shoots and Roots in Rooting Media

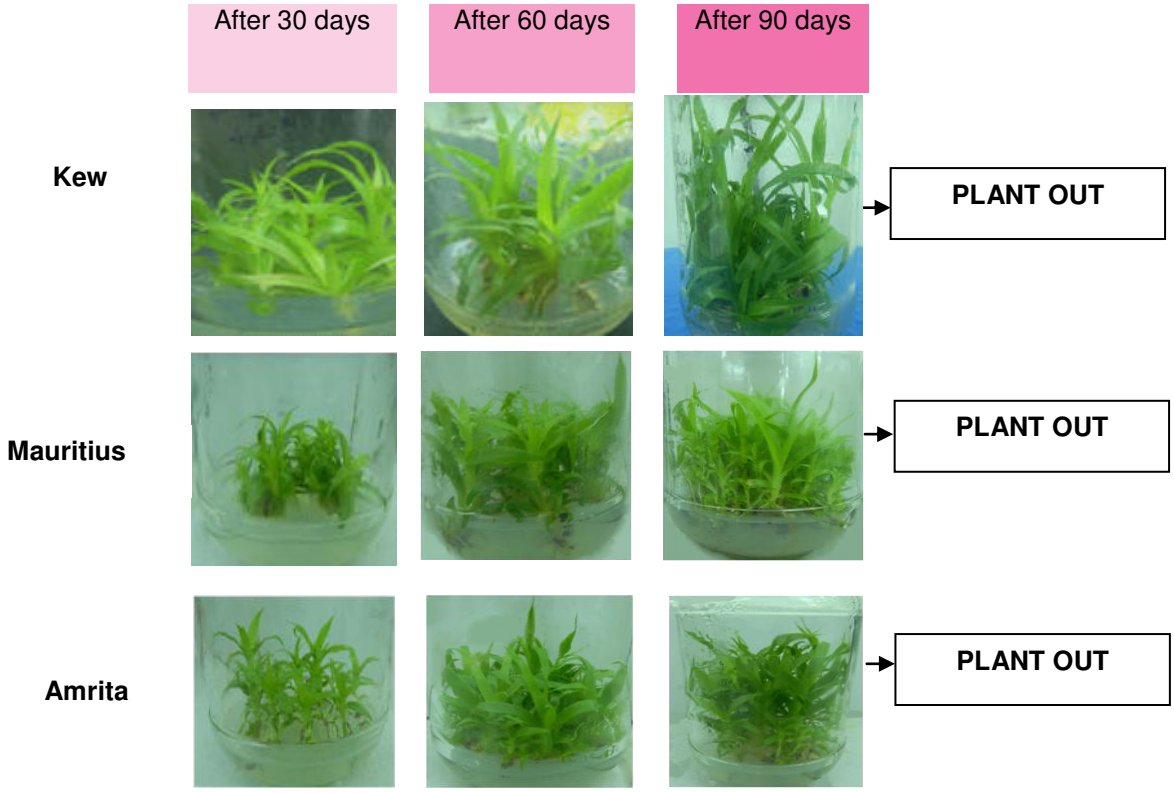


Fig. 31. Rooting of Kew, Mauritius and Amrita

6. Planting out and Hardening

Fully rooted *in vitro* plants with 4-5cm shoot length are selected for planting out. Healthy plants were treated with Pseudomonas 20g/l for 30 minutes and planted in potting mixture. Contaminated plants were treated with 2.5g/l SAAF for 30 minutes and planted.

Potting mixture is made by mixing 100 kg Soil+10 kg Cow dung+1 kg Neem Cake. The mixture is thoroughly mixed and irrigated well. Potting mixture is covered for 4-5 days and Trichoderma (1-2 kg) is added. It is again mixed and irrigated well. Mixture is again covered. For one week it is irrigated and mixed at 2 days interval. This mixture is further used for planting.

Plants are first grown in mist chamber for acclimatizing with climate outside the lab **(Primary Hardening)**. After 2-3 weeks' time, they are moved to green house to get adjusted with field conditions **(secondary Hardening).** Plants are planted in potting mixture. After 12 months the plants are ready for field planting.

For the planting of a mature hardened plant to the field, we require 20-22 months of duration from the date of inoculation, and we get more than thousand plants from an explant that is inoculated in the artificial media.

Fig.32. Plant Out of MD-2, Mauritius and Amrita

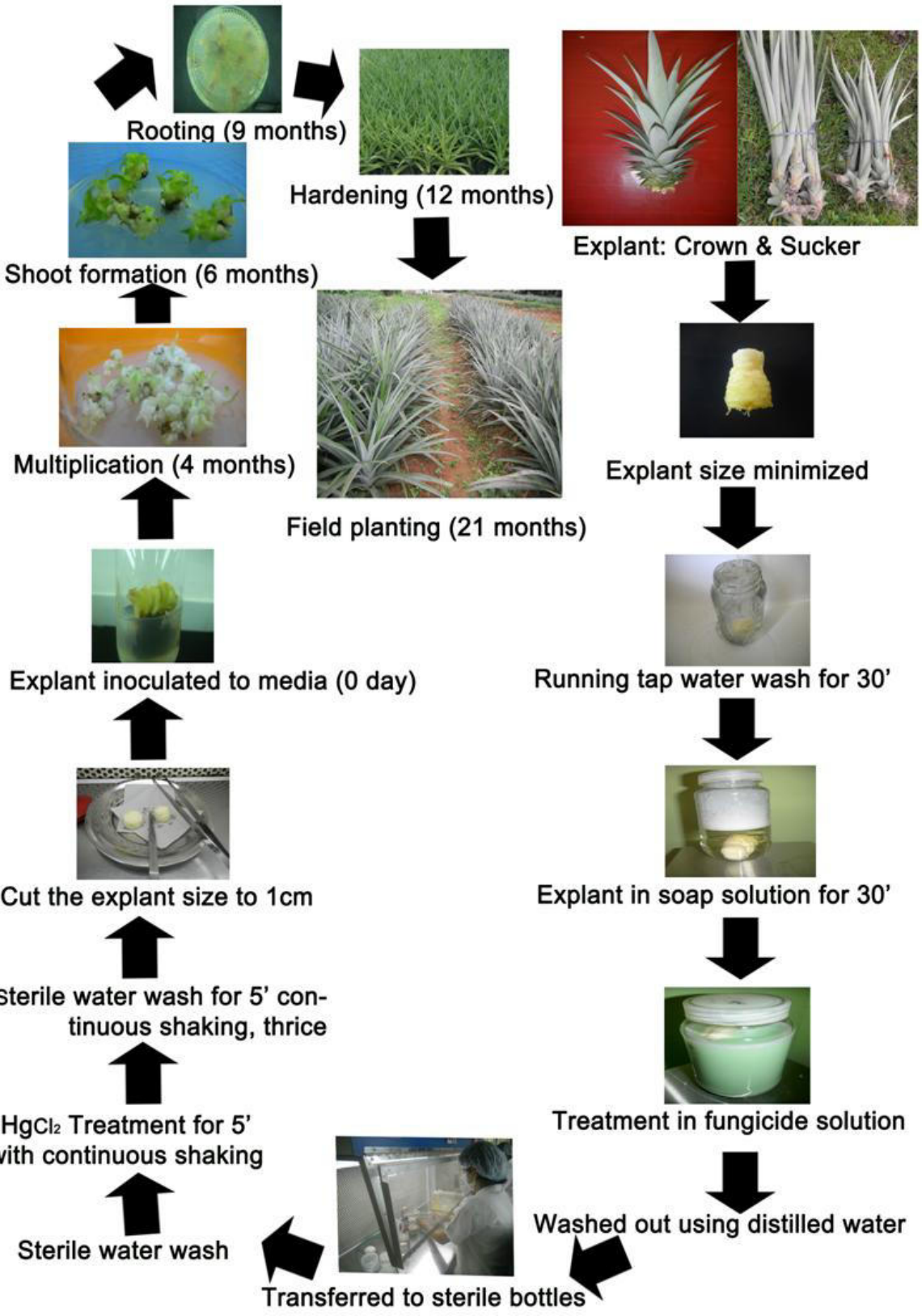
Tentative production estimate

Table 5. Tentative production estimate of pineapple tissue culture plants (Nos)

Responding Explants	Periods (Months) (If recovery ≥ 90%)						
	12	15	18	21	24	27	30
1	400	900	1300	1600	2000	2200	2400
5	2000	4200	6200	8000	10000	11000	12000
10	4000	8200	12200	16000	20000	22000	24000
50	20000	40200	60200	80000	100000	110000	120000
100	40000	80200	120200	160000	200000	220000	240000

One Biotechnologist & 6 racks for every 25000 TC plants (2500 bottles of 10 plants)
5750 TC plants (575 bottles of 10 plants) for every rack.

Tissue Culture of Pineapple



11

Passion Fruit Tissue Culture

Micropropagation of Passion Fruit

Micro propagation studies of Passion fruits are in the initial stages and the standardization of the fresh inoculation of explants is completed. As in the case of every tissue culture protocol it also involves the same stages. Only the difference is the type of explant used.

Explant source & Type: Yellow and Purple Passion fruit seedlings in the field of Pineapple Research Station, Vazhakulam. Meristematic shoot tips, nodes and young leaves are used for micro propagation.

Fresh Inoculation

The selected explants are surface sterilized. The protocol is as follows. Chop off the unwanted exposed portions of the plants. Keep the explants in running water for 30 minutes. Wash with Tween 20 to clean the explants for 20 minutes. Place them in running water till the complete removal of soap. Treat them in Bavistin (0.1%) and Indofil (0.1%) fungicide combination for 30 minutes. Wash the explants with distilled water. Further dip the explants in 70% alcohol. In the LAF again treat the explants in 70% ethanol. After that transfer the explants in 0.1% $HgCl_2$ (w/v) for 5 minutes followed by 3 rinses of 5minutes each in sterile distilled water. Resize the nodes and leaves and dip in antibiotic (Gentamycin 2ml/l) solution and inoculate to the media. The cultures are carefully tightened with cotton plug and properly labeled before transferring to incubation room at 25-27^{o}C temperature.

The cultures are green after seven days. Young leaves develop from the node after 14 days. Callus formation occurs after 21 days.

The subculture stages and rooting are under preliminary stages of study.

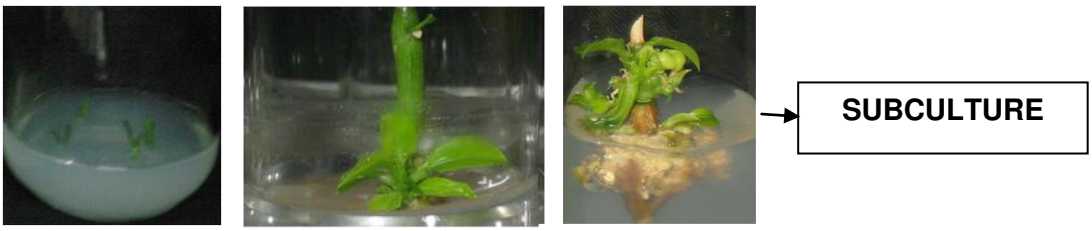


Fig. 33. Stages in initiation of Passion fruit Nodal culture

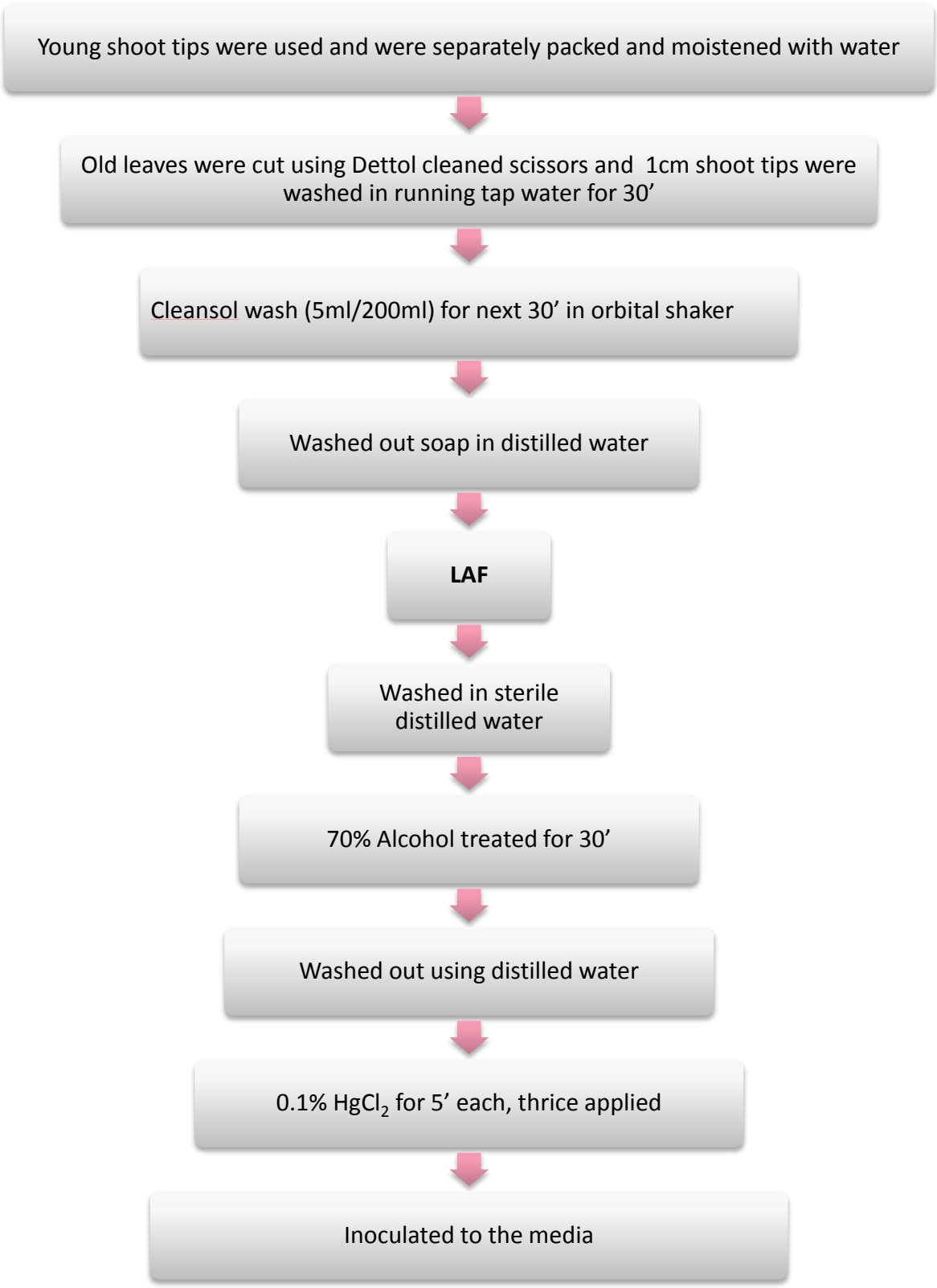


Fig. 34. Protocol for Shoot tip Tissue Culture of Passion Fruit

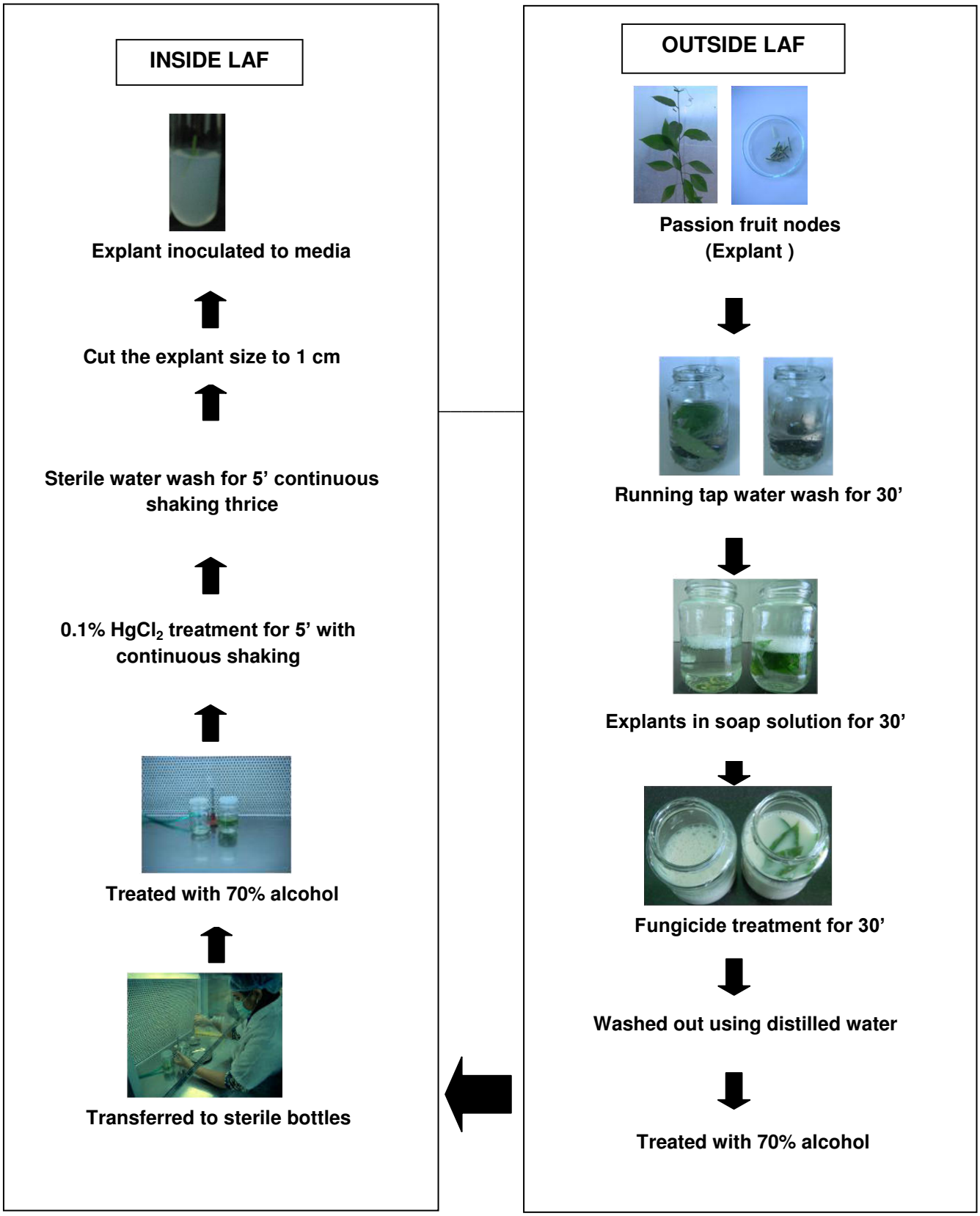


Fig. 35. Steps in fresh inoculation of Passion fruit Nodes

12

Banana Tissue Culture

Micro propagation of Banana

We are mainly focusing on the tissue culture studies of five varieties of banana namely Nendran, Poovan, Njali Poovan, Robusta and Red banana. Other varieties namely Grand Naine, Swarna Mukhi are also studied here. Explants used are shoot tips and inflorescence of banana. Micro propagation of Banana includes three major stages:

1. **Initiation (Fresh inoculation)**
2. **Mass Production (Multiplication)**
3. **Rooting and Hardening (Plant out)**

1. (a) Fresh Inoculation of Banana shoot tip (sucker)

Six months old healthy disease free banana suckers are suitable. Wash the explants thoroughly in tap water, remove roots and leaf sheaths and cut the basal portion of the corm and trim to a size of 12x12x15 mm. Keep the explants under running tap water for 30 minutes, soak them in teepol (detergent) for 30 minutes and shake in orbital shaker, wash with distilled water to remove the teepol particles, treat with fungicide for 30 minutes and wash with distilled water to remove traces of fungicide. Further treat them with antibiotic (2ml/l) for one hour. Wash out it with distilled water. Transfer them to laminar air flow chamber for surface sterilization. Inside the laminar air flow chamber, treat the explants with 70% ethanol (2 minutes) and then with 0.1% Mercuric chloride for 5 minutes followed by three rinses of 5 minutes each with sterilized distilled water. Resize the explants to a final size of 8x8x10 mm in sterile conditions of the inoculation chamber, and inoculate on to culture media and incubate at 25±2^{o}C in dark for 21 days.

The fresh inoculated banana shoot tip (sucker) cultures are observed for a cycle of 21 days. After 7 days bulging starts along with elongation of central bud. Within 14 days buds develop to sprout. After 21 days the bud number increases from 0 to 4. No callus formation occurs. Use the same media for media change of the cultures for increase in buds number and continue till three media changes.

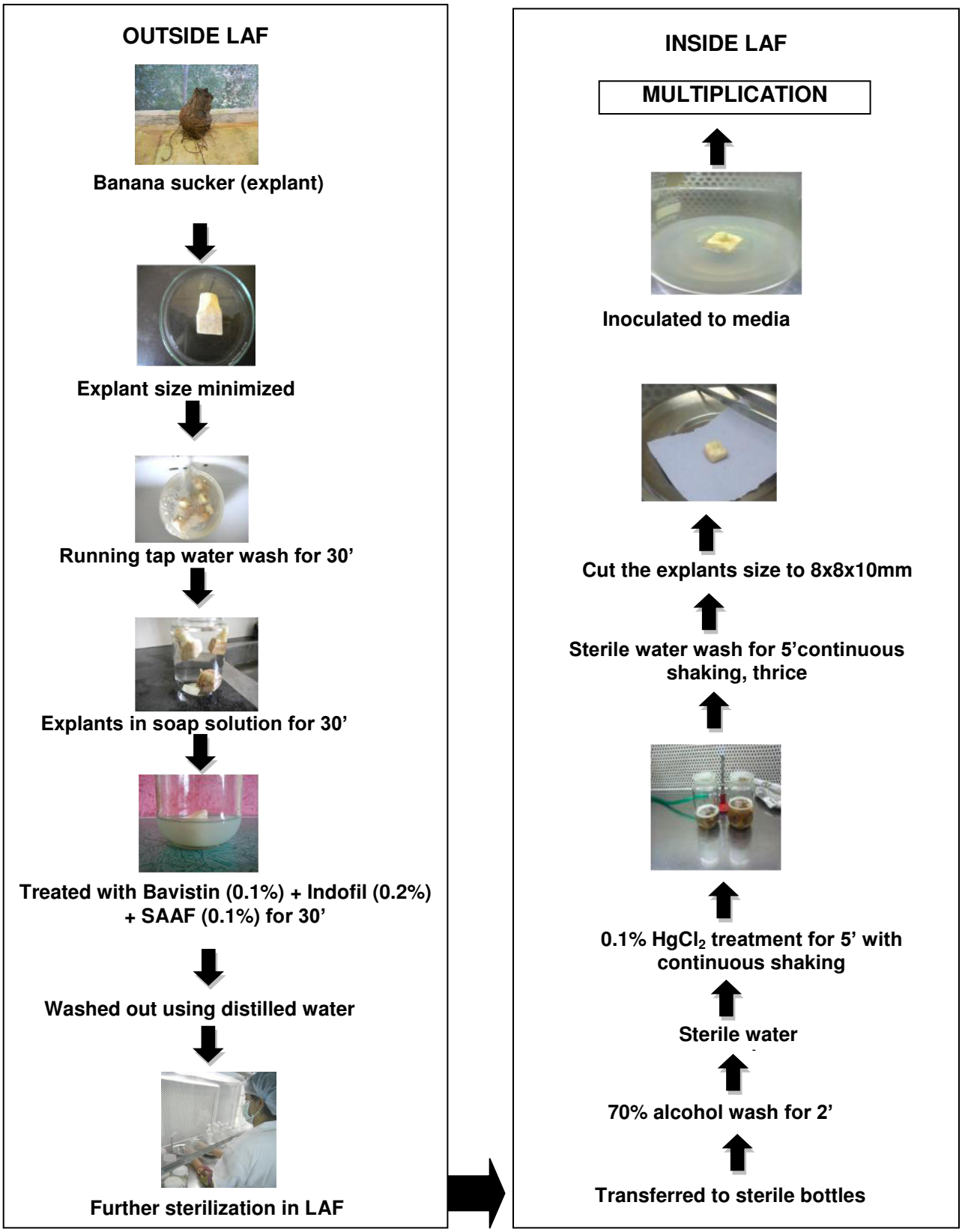


Fig. 36. Fresh Inoculation of Banana shoot tip

1. (b) Fresh Inoculation of Banana Inflorescence

Banana inflorescence is a perfect choice as explant since it is areal and comes across with air borne microbes only.

Collect the healthy disease free explants from farmers' fields and go for the following sterilization procedures. The explant is separated from the mother plant after the formation of all the female flowers. Remove the bracts from the male flowers until they become too small (3 cm in length).

Place the explants under running tap water for 30 minutes, soak it in teepol (detergent) for 30 minutes and shake continuously, treat with fungicide (Bavastin 0.1%, Indofil 0.1% and SAAF 0.05%) for 30 minutes and wash with distilled water to remove fungicide. Further transfer to laminar air flow chamber for the remaining surface sterilization procedures.

Inside the laminar air flow chamber, treat the explants with 70% ethanol (2 minutes) and then with 0.1% Mercuric chloride for 5 minutes followed by three rinsing of 5 minutes each with sterilized distilled water. Trim the explants to a final size of 8x8x10 mm in sterile conditions of the inoculation chamber, and inoculate on to culture media.

Bulging of cultures start after seven days and further new buds develop in upcoming days and after 21 days it increase even up to 10 numbers. Media changes can be applied thrice here for further bud proliferation.

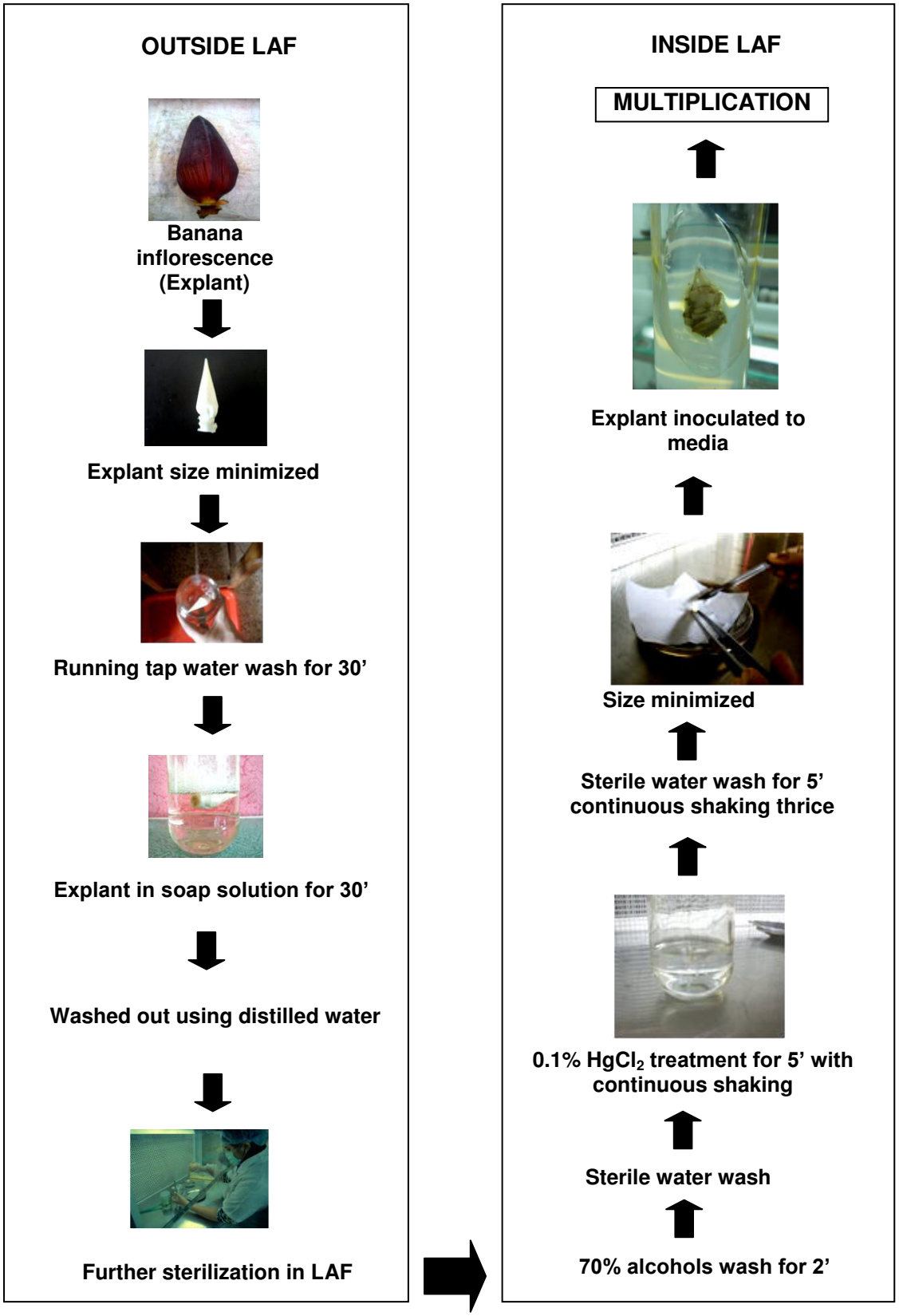


Fig. 37. Fresh inoculation of banana inflorescence

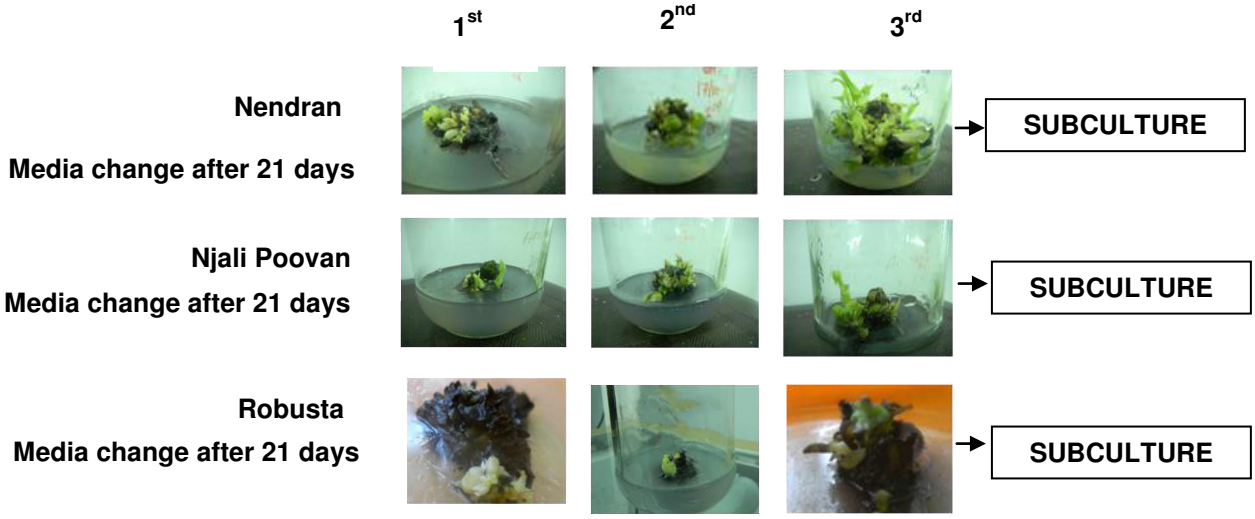


Fig. 38. Media Change of Banana Inflorescence

2. Multiplication

2. (a) Subculture of Banana shoot tip (sucker)

Fresh inoculation medium only increases bud number. Multiplication of cultures is effective through callus regeneration. Hence the cultures are to be inoculated to a medium fortified with auxin especially IBA. The buds along with the rhizome portion are cut to a smaller size and inoculate to the callus regeneration medium. This commences subculture stage. After enough cultures with shoots are obtained they are ready to be cultured to rooting media for root formation.

MS+3mg/l IBA+1.5mg/l BA is the medium effective for the entire banana shoot tips except for Poovan. Increased bud formation up to 16 buds in case of red banana was observed after 3rd subculture. MS+2mg/l BA is the medium found effective only in case of Poovan.

2.(b) Subculture of Banana Inflorescence

MS+3mg/lBA medium is not effective for multiplication. Hence Banana inflorescence cultures are sub cultured to MS+3mg/lIBA+0.5mg/lBA media for increase in buds number and callus formation. This stage is continued till 6th subculture. After enough cultures are obtained they are sub cultured to rooting media for root formation

MS+3mg/l IBA+1.5mg/l BA medium is effective exclusively for banana inflorescence. 22 shoots at 3rd subculture are observed to a maximum in Nendran.

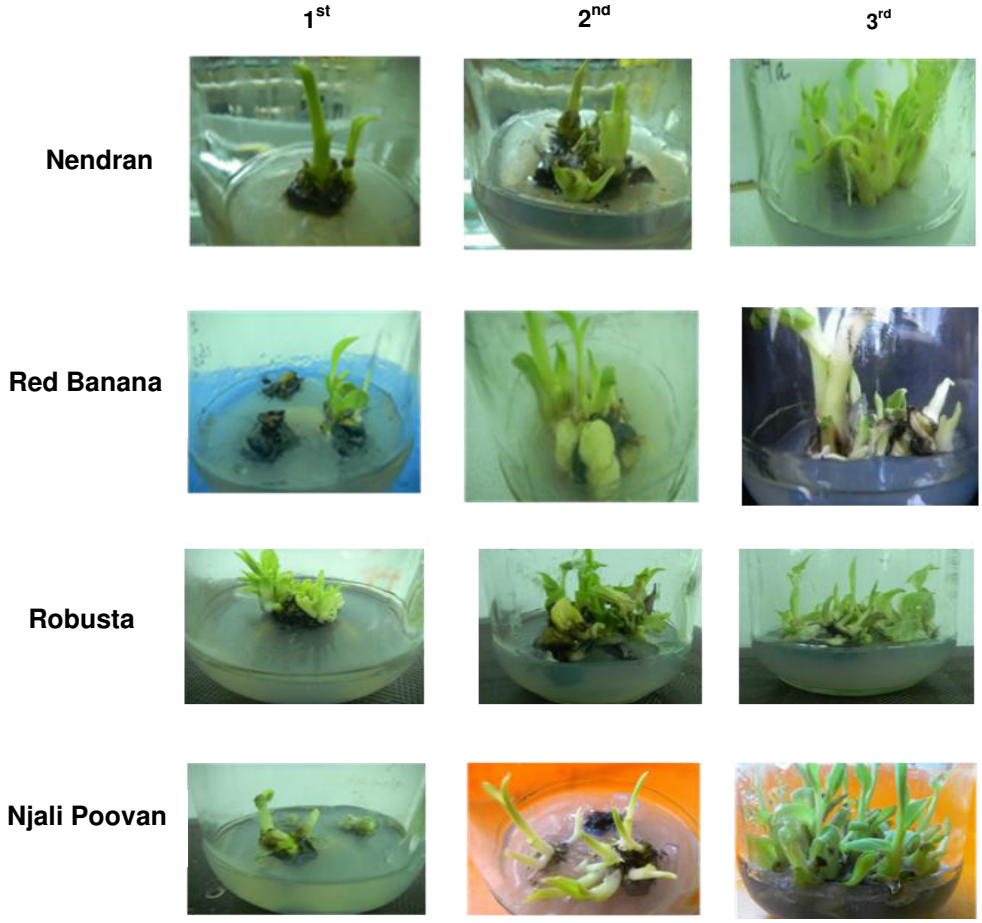


Fig. 39. Shoot production in sequential subculture of banana shoot tip (sucker) after 21 days

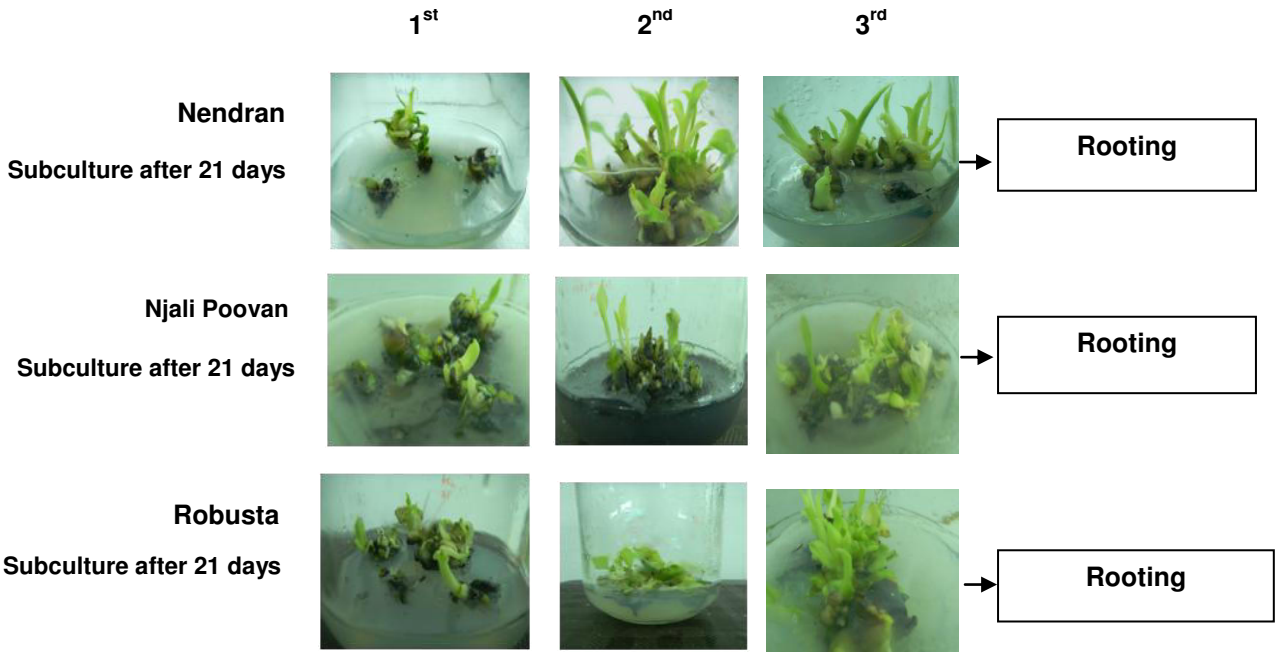


Fig. 40. Subculture of banana inflorescence

Rooting

Explants with enough leaves and shoots are separated carefully and transferred to rooting medium and incubated at 25±2°C.

HMS (Half strength MS medium) is the medium giving favorable performance.

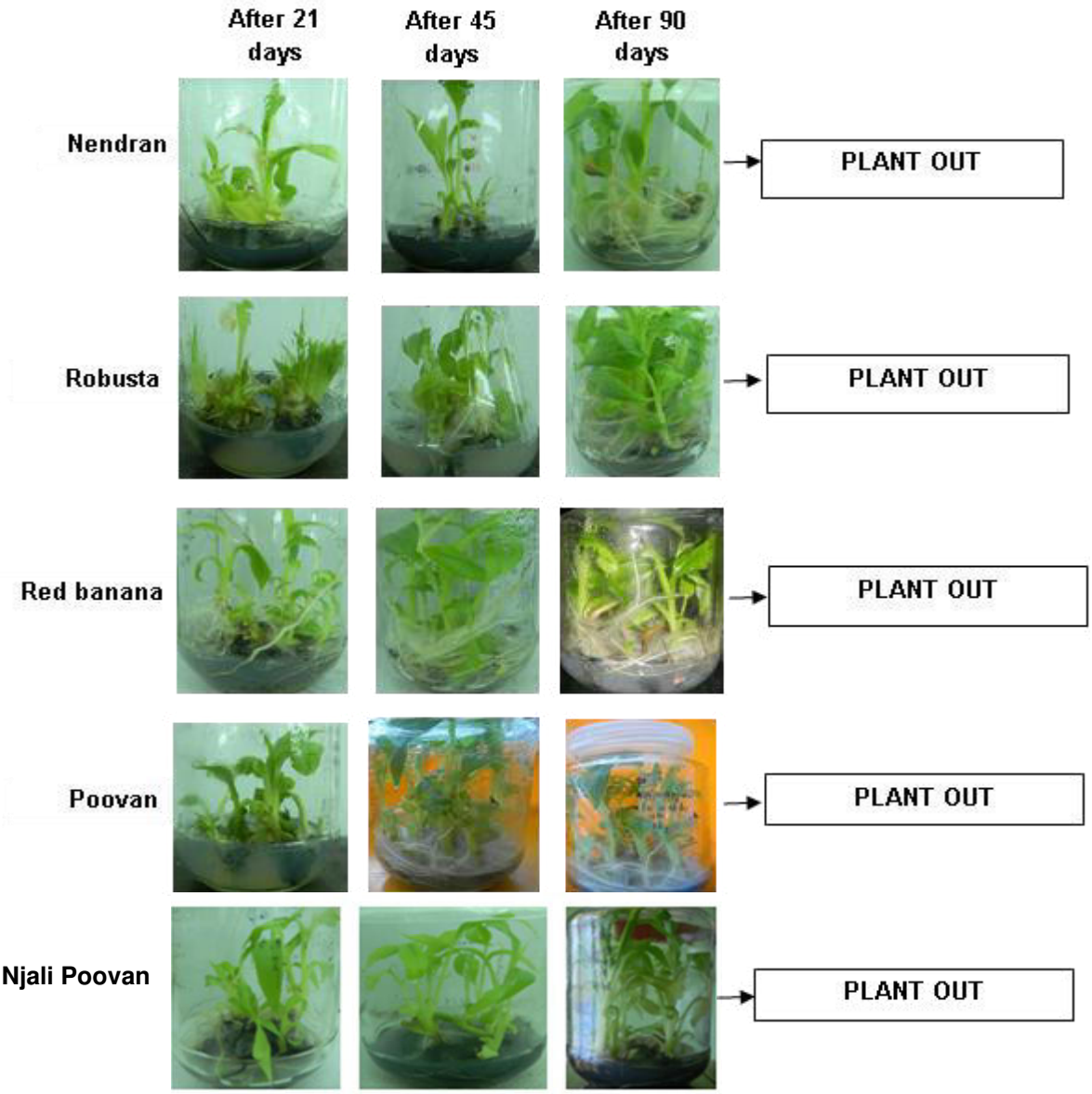


Fig. 41. Rooting of banana

Hardening of Banana

Fully rooted plants *in vitro* are selected for plant out. Plants are first grown in mist chamber for acclimatizing with climate outside lab. After 2 to 3 weeks' time they are moved to green house to get adjusted with field conditions. Healthy plants are treated with Pseudomonas 2g/l for 30 minutes and planted. Fungal contaminated plants are treated with 2g/l SAAF for 30 minutes and planted. Plants are planted in potting mixture.

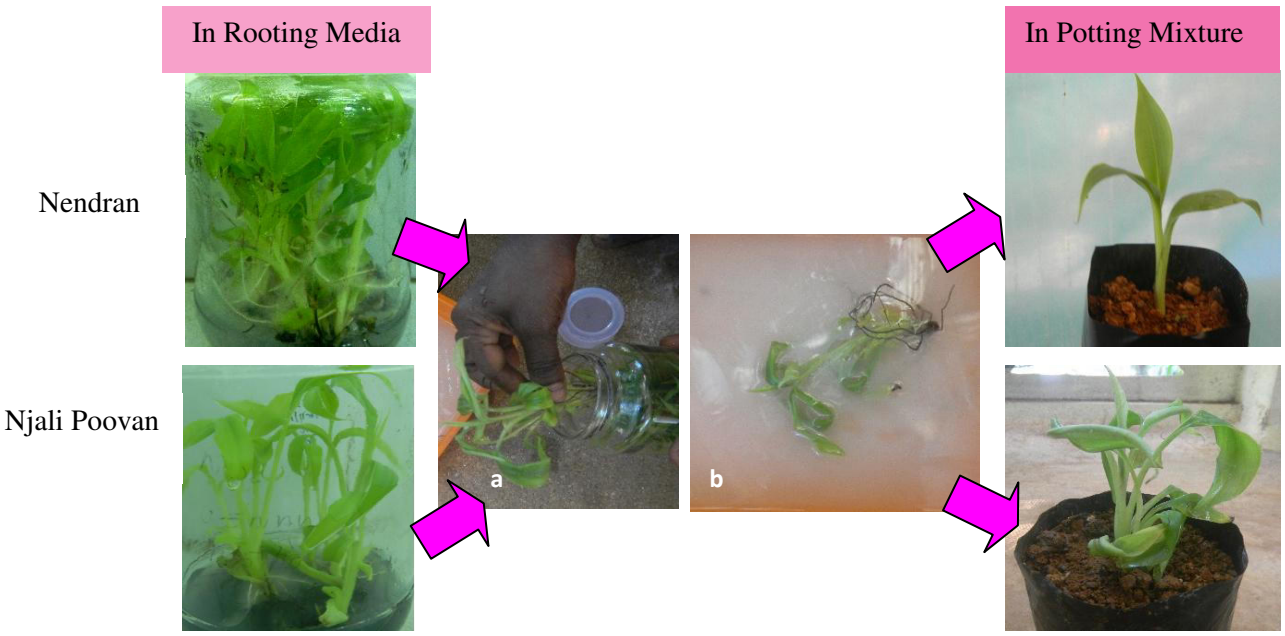


Fig. 42. Hardening Protocol a. Tissue Culture Plants washed in tap water
b. Plants dipped in Pseudomonas

Nendran **Robusta** **Njali Poovan**

Fig. 43. Banana Tissue Culture Plants after one month

For the planting of a mature hardened plant to the field, we require 9 months duration from the date of inoculation, and we can produce more than thousand plants from an explant that is inoculated in the media.

Table 6. Tentative production estimate of banana tissue culture plants (Nos)

Responding Explants	Period (Months) (If recovery ≥ 90%)			
	9	12	15	18
1	25	500	800	1200
5	125	2500	4000	6000
10	250	5000	8000	12000
50	1250	25000	40000	60000
100	2500	50000	80000	120000
500	12500	250000	400000	600000
1000	25000	500000	800000	1200000

One Biotechnologist & 6 racks for every 25000 TC plants (2500 bottles of 10 plants) 5750 TC plants (575 bottles of 10 plants) for every rack.

Diagrammatic Representation of Micro propagation of Banana

Selection of mother plant
(0 day)

Banana Inflorescence

Preparation of explants

Fresh inoculation (0 day)

Multiplication (90 days)

Field planting (9 months)

Secondary hardening
(8 months)

Planting out & primary
hardening (7 months)

Rooting (6 months)

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13

Tips for Contamination free Tissue Culture Lab

Precautions to be taken for the maintenance of tissue culture lab

1. Aseptic Techniques

For the best results in tissue culture, we want to work to keep microbial (bacteria, yeast and molds) contamination to a minimum. To do this, there are certain things we must be aware of. They are

- Work in a culture hood set-aside for tissue culture purposes. Most have filtered air that blows across the surface to keep microbes from settling in the hood. Turn off the UV/antimicrobial light and turn on the hood 30 minutes prior to entering the hood
- Wear short sleeves or roll your sleeves. Tie long hair back and remove rings and watches
- Wash hands with soap and water before beginning the procedure and rewash if touch anything that is not sterile or within the hood
- Spray down the hands, work surface and anything that will go into the hood with 70% ethanol. Re-wipe at intervals if working for a long time in the hood. This will reduce the number of bacteria and molds considerably.
- Do not breathe directly into the cultures, bottles of media. This also means to keep talking to a minimum. No mouth activities.
- Keep bottles and flasks closed when we are not working with them. Avoid passing arm or hands over an open bottle.
- Use only sterilized pipettes, plates, flasks and bottles in the hood for procedures.
- Take special precautions with the sterile pipettes. Remove them from the package just before use. Never mouth-pipette, use the pipetting aid. Change pipette for each manipulation. If the tip of the pipette touches something outside of the flask or bottle, replace with a new one. Never use a pipette twice.
- Spray spirit in LAF before and after the work

2. Safe Work Practices

- Maintain a clean work area.
- Know the locations of the nearest emergency exit and safety equipment (shower, eye wash)
- Keep maximum cleanliness by each and every worker

3. Good laboratory practices

- No smoking, no food or beverages in the lab
- Required PPE (Personal Protection equipment's) must be worn while in the lab
- Lab benches must be cleaned, disinfected or decontaminated after work is completed
- *Do not use hoods for storage*
- Broken glass containers are available & use. Glassware is free from crack, breaks or chips
- Heavy objects are confined to lower shelves

Precautions to be taken for a sterile tissue culture lab

- Regular cleaning of Tissue culture Lab using Dettol/Lysol
- Give more attention in media preparation when measuring, mixing, pouring, sterilization and transfer to Tissue Culture laminar air flow
- Media for culturing as well as sub culturing should be sterilized in autoclave, and once sterilized should not be again sterilized; it will change the composition of ingredients.
- The room should have a double door and it should be kept closed at all times
- All culture vessels including pipettes should be sterilized by dry heat in Hot Air Oven at 160-180^{o}C
- Staff should wear apron/lab coat, gloves, mask (on head and mouth) while working inside Tissue culture lab
- Tie hair properly and wear minimum ornaments and accessories. Cut the nails regularly and don't use nail polishes.
- Used glass wares and media must be decontaminated by autoclaving before washing and disposal.
- The platform of LAF and hands of staff working must be wiped with 70% alcohol
- Routine close observation of tissue culture flask and bottles are necessary (examine the presence of white/cream colored spreading or any colored mycelia growth)
- Remove all infected flasks immediately seeing any kind of contamination
- Lab coat, gloves and mask must be autoclaved after washing & drying
- Before all tissue culture operation the laminar air flow should be pre-sterilized by putting
- U-V light for 45min.The platform must be wiped with spirit or 70% alcohol every corner
- Media preparation room, inoculation and culture maintenance room should be fumigated with formalin at regular intervals. All the doors, ventilations, electric circuits should be off before fumigation
- Keep maximum cleanliness by each and every worker
- The doors to the tissue culture rooms are to be kept shut all times
- Staff should wear a lab coat and acid resistant gloves
- All culture vessels are washed with detergent, rinsed with tap water and then finally with distilled water.
- Washed glasswares must be sterilized by dry heat in an oven at 160-180^{o}C for 1.5 hours.
- Used glasswares should be autoclaved before washing to remove traces of agar and destroy infection
- Label EVERYTHING
- After pouring medium the culture vessels should be plugged with non-absorbent cotton or caps and finally autoclaved at a pressure of 15lb for 15 minutes in an autoclave. Some types of plastic wares and instruments like pipettes are also autoclavable. Instruments like forceps, scalpels, needles, spatula etc. should be first dipped in spirit, sterilize over flame and then leave for drying
- Growth regulators like GA_3, Zeatin, Urea, ABA, certain vitamins are heat liable hence filter sterilized by passing through a membrane filter of 0.45µ or lower pore size.

- The platform of the laminar flow cabinet, hands of the operator etc should be sterilized by wiping with 70% alcohol. Wipe off anything you intend to use in the hood.
- If you spill anything in the hood, clean up immediately to prevent cross contamination and damage to the work place
- Standardized surface sterilization protocols should be followed depending on the type of explant
- Laminar Air Flow Chamber is pre-sterilized by ultraviolet radiation for 45 minutes. It must be properly wiped with 70% spirit before UV Sterilization and starting the inoculation work.
- Tissue culture rooms and labs should be kept clean and disinfected
- The culture room and inoculation room must be fumigated with formalin at regular intervals against bacterial and fungal contaminations
- All the doors and windows should be closed and all electric circuits should be off before the process of fumigation.
- Examine all tissue culture flasks for any contamination (tiny dots of bacteria or stings of hyphae from fungi. Remove all infected flasks into an appropriate part of the laboratory.
- The contaminated culture flasks are decontaminated using autoclave and they are dipped in Chromic acid for a night and washed in detergent the very next day. Further washed in distilled water and kept for drying.
- Any sterile media left after culturing must be placed in sterile conditions.
- Put coats to laundry once in a week.

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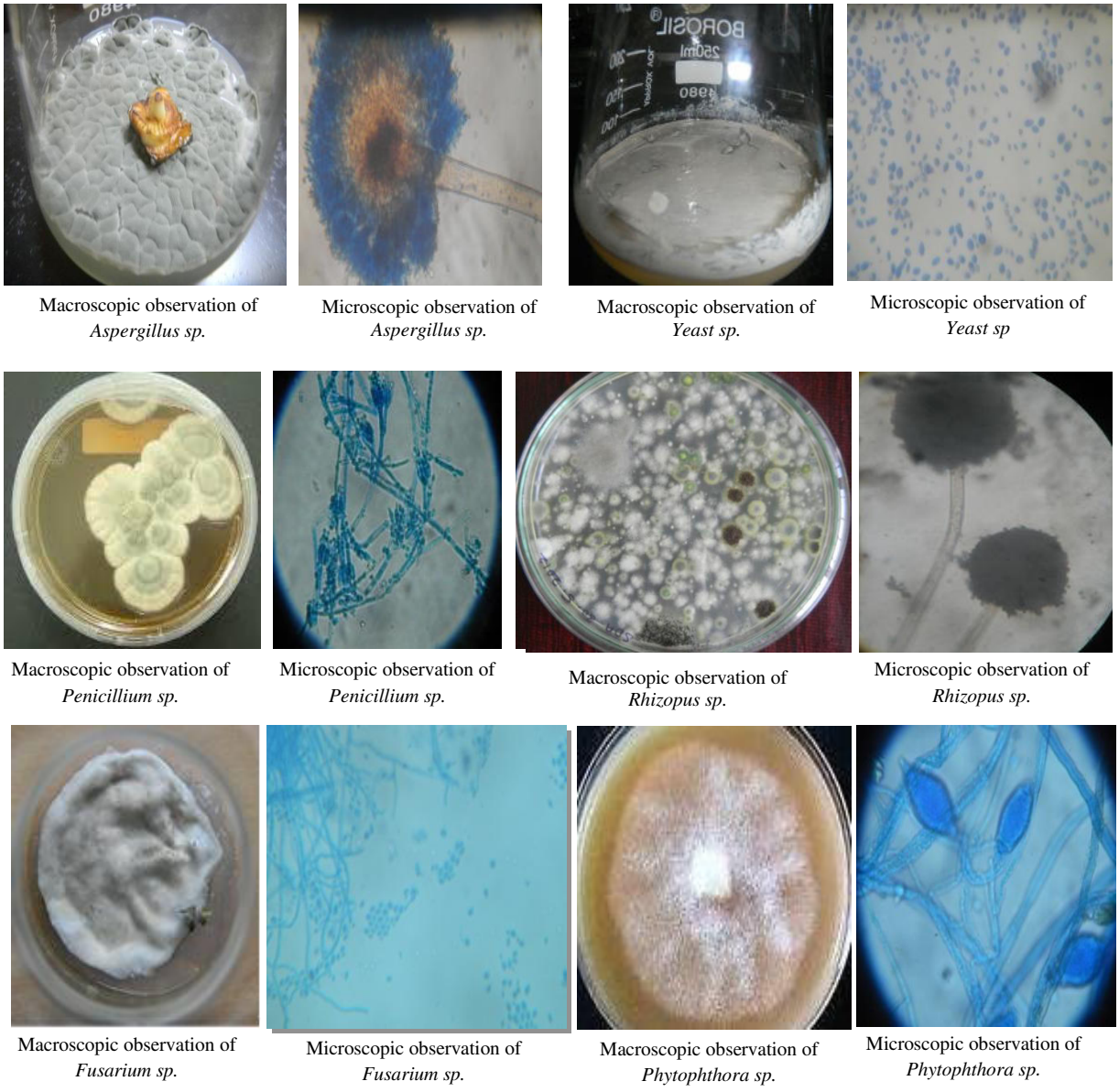
Common Tissue Culture Contaminants

Fungi are the main contaminants in tissue culture lab. About 90% introduction of those microbes are during fresh inoculation. Soil borne pathogens and air borne pathogens are the main contaminants then. Strict sterilization procedures must be followed during fresh inoculation stage.

At subculture stages, the working environment and the lab worker could be responsible for the contaminants. The major contaminants are Aspergillus, Penicillium, Yeast and bacteria.



Macroscopic observation of *Aspergillus sp.*

Microscopic observation of *Aspergillus sp.*

Macroscopic observation of *Yeast sp.*

Microscopic observation of *Yeast sp*

Macroscopic observation of *Penicillium sp.*

Microscopic observation of *Penicillium sp.*

Macroscopic observation of *Rhizopus sp.*

Microscopic observation of *Rhizopus sp.*

Macroscopic observation of *Fusarium sp.*

Microscopic observation of *Fusarium sp.*

Macroscopic observation of *Phytophthora sp.*

Microscopic observation of *Phytophthora sp.*

Fig. 44. Macroscopic and Microscopic Observations of Tissue Culture Contaminations

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Mass Production of Tissue Culture Plants

Tissue culture plants mass produced are sold as hardened plants and bottle plants. Banana and MD-2 plants are cultured as 10 plants per bottle and the fully grown plants are ready for sale. The hardened plants in green house having 8-10 cm height are also suitable to be sold.

Fig. 45.(a),(b) & (c) Tissue culture banana plants, pineapple and passion fruit plants in rooftop nursery (d) Ten plants per bottle – MD2 (e) Ten plants per bottle – Banana

Fig. 46. Plant Sale (a) sale of polybag plants (b) sale of bottle plants (c) sale of MD2 tissue culture plants separated from polybags

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Plant Molecular Studies

DNA Isolation of Plant Samples

Samples are leaves from Kew, H-1(nursery) and H-1(Field). Grind 0.5g fresh leaf material using pre-chilled mortar & pestle in presence of 5ml extraction buffer and 50µl β–mercaptoethanol. Transfer the homogenate into a 15ml centrifuge tube. Incubate the sample at 65ºC for 20 minutes with occasional mixing by gentle inversion. Add equal volume of chloroform: isoamyl alcohol (24:1) and mix by inversion and incubate at 65ºC for 5 minutes. Centrifuge at 10,000 rpm for 15 minutes at 4ºC. Remove the aqueous phase with a wide – bore pipette, transfer to a clean tube and added 0.6 volume of chilled isopropanol and mixed by quick gentle inversion till DNA is precipitated. Keep at -20ºC for half an hour. Centrifuge at 10,000 rpm for 10 minutes at 4ºC. Discard the supernatant and wash the DNA pellet in 70% ethanol. Centrifuge at 10,000 rpm for 5 minutes at 4ºC. Supernatant is discarded and air dried the pellet, dissolve in 50µl sterilized distilled water. Store at -20ºC. Dissolve the DNA in 50µl sterile water and store at -20ºC.

Amplification of Plant DNA using PCR Technique

Polymerase Chain Reaction is widely held as one of the most important inventions of the 20th century in molecular biology. Small amounts of the genetic material can now be amplified to be able to identify, manipulate DNA, detect infectious organisms, detect genetic variations, including mutations, in plant genes and numerous other tasks.

PCR involves the following three steps: Denaturation, Annealing and Extension. First, the genetic material is denatured, converting the double stranded DNA molecules to single strands. The primers are then annealed to the complementary regions of the single stranded molecules. In the third step, they are extended by the action of the DNA polymerase. All these steps are temperature sensitive and the common choice of temperatures is 94^{o}C, 60^{o}C and 70^{o}C, respectively. Good primer design is essential for successful reactions.

Here, the PCR technique is used to amplify the isolated DNA from pineapple.

Materials: PCR kit, thermal cycler, sample DNA, Micropipette.

Reaction mixture: A

1. Sterile water - 19 µl
2. 10X assay buffer- 2.5 µl
3. 10 µm dNTP- 1.5 µl
4. Forward primer-0.5 µl
5. Reverse primer- 0.5 µl
6. Template DNA- 10X diluted- 0.5 µl
7. Taq DNA polymerase- 0.5 µl

Total reaction mixture- 25 µl

Reaction mixture: B: - sample DNA provided with the kit.

1. Sterile water - 38 µl
2. 10x assay buffer- 5 µl
3. 10 µm dNTP - 3 µl
4. Forward primer-1 µl
5. Reverse primer- 1 µl
6. Template DNA- 10x diluted- 1 µl
7. Taq. DNA polymerase- 1 µl

Total reaction mixture- 50 µl

Keep the reaction mixture in ice bath.

Reaction condition

Initial denaturation	- 94ºC	1 minute	
Denaturation	- 94ºC	2 minute	
Annealing	- 48ºC	30 seconds	→ for 30 cycles
Extension	- 72ºC	1 min	
Final extension	- 72ºC	2 min	

Lid temperature 105ºC, hold temperature 4ºC

Agarose Gel Electrophoresis

After PCR amplification, 5 µl gel loading buffer is added to the reaction tubes and mixed well. 15 µl samples are loaded in 0.8% agarose gel prepared in 1X TAE buffer. The age set up is done and run for approximately 2 hours. (Applied Voltage: 80 volts DC). After AGE is completed, the gel is observed in UV Transilluminator.

DNA bands can be observed as orange bands.

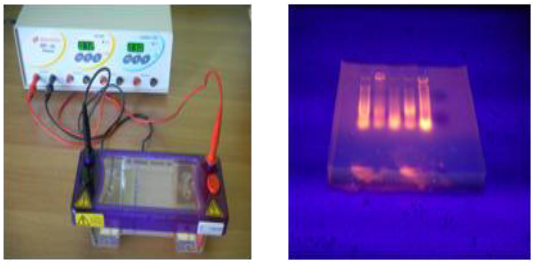

Fig. 47. Electrophoresis Unit and DNA bands obtained in Gel Doc

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Price: ₹ 100