S.YBSc.Credit pattern Term II BO 242

Botany paper II.Plant Biotechnology

Chapter 2- Plant Tissue culture

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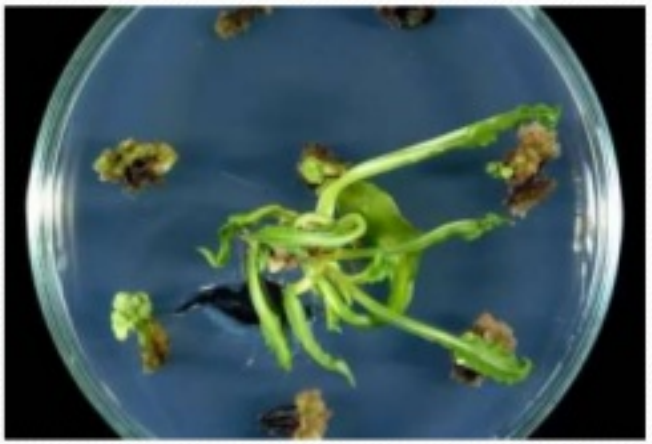
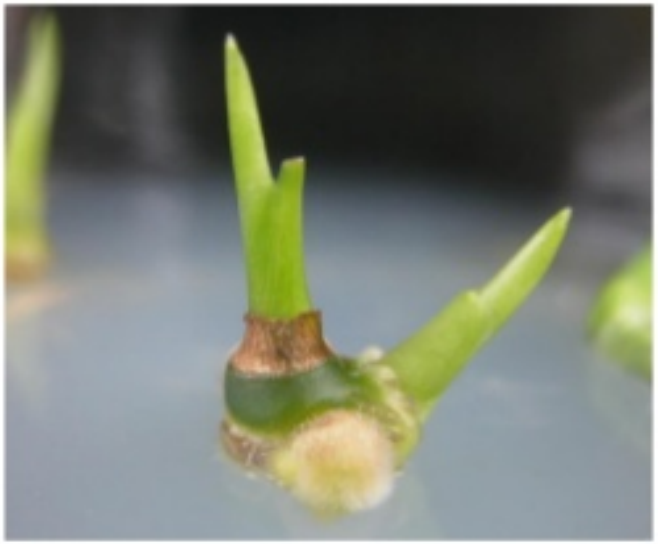
INTRODUCTION

- Plant tissue culture (PTC) is a form of asexual propagation of plants under laboratory conditions.
- Plant tissue culture is a technique with which plant cells, tissues or organs are grown on artificial nutrient medium, either static or liquid, under aseptic and controlled conditions.

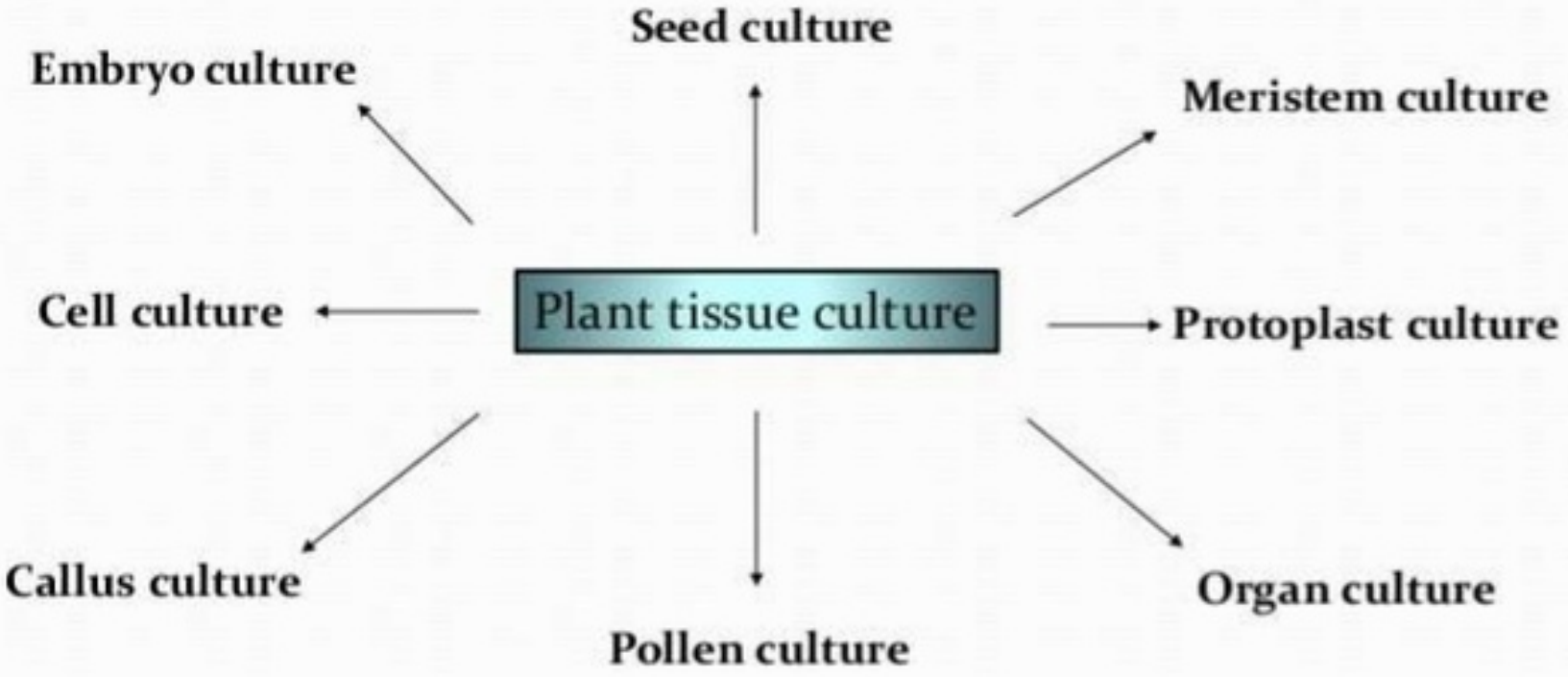


DEFINITION

- **Tissue culture** is *in vitro* cultivation of plant cell or tissue under aseptic and controlled environmental conditions, in liquid or on semisolid well defined nutrient medium for the production of primary and secondary metabolites or to regenerate plant.



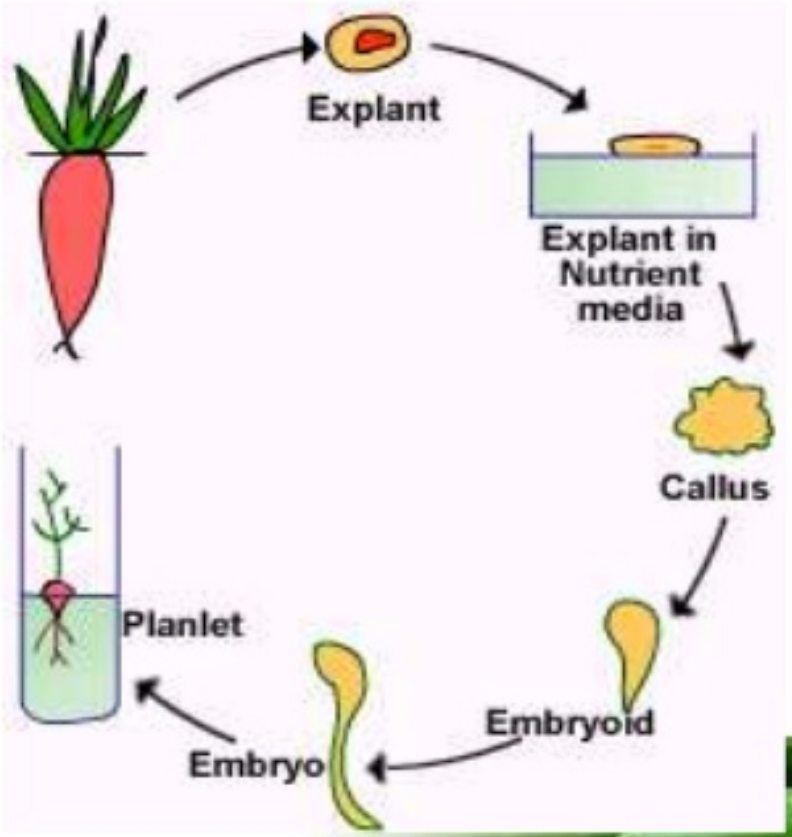
TYPES OF PTC


Seed culture
Embryo culture
Meristem culture
Cell culture
Plant tissue culture
Protoplast culture
Callus culture
Pollen culture
Organ culture

2.1.1Cellular Totipotency

- Totipotency is the genetic potential of a plant cell to produce the entire plant.
- Professor Gottleib Heberlandt (1902) a German Botanist first use the term Totipotency. He is known as Father of Plant Tissue Culture.
- After providing nourishment, growth factor , environmental condition the cell multiplies & developed tissue or organ & grow as new plant
- In plant root shoot tip region,embryo,cambium are meristematic & totipotent but somatic or vegetative cells are also totipotent
- In animals only zygote,initial embryo cells are totipotent

CONCEPT OF TOTIPOTENCY



- As cell divide mitotically, they do so eqautionally to produce daughters cells.
- G.Haberlandt's claimed that one day it could be possible to rear plants from isolated would be rarely surviving cells of flowering plants.
- He also sated that out of surviving somatic cells artificial embryos would be reared asexually
- Therefore every cell within the plant has a potential to regenerate into a whole plant.

- **Expression of Totipotency in Culture:**
- The basis of tissue culture is to grow large number of cells in a sterile controlled environment. The cells are obtained from stem, root or other plant parts and are allowed to grow in culture medium containing mineral nutrients, vitamins and hormones to encourage cell division and growth. As a result, the cells in culture will produce an unorganised proliferative mass of cells which is known as callus tissue.

Cellular totipotency

- **Cytodifferentiation**
  - **Cell differentiation, mainly emphasis on vascular differentiation, tracheary element differentiation, etc.**
- **Dedifferentiation**
  - The phenomenon of mature cells reverting to a meristematic state and forming undifferentiated **callus** tissue.
- **Redifferentiation**
  - The ability of the component cells of the callus to differentiate into a whole plant or organ.

High ratio of cytokinin to auxin = shoot development (caulogenesis).
Low ratio of cytokinin to auxin= roots development (rhizogenesis).

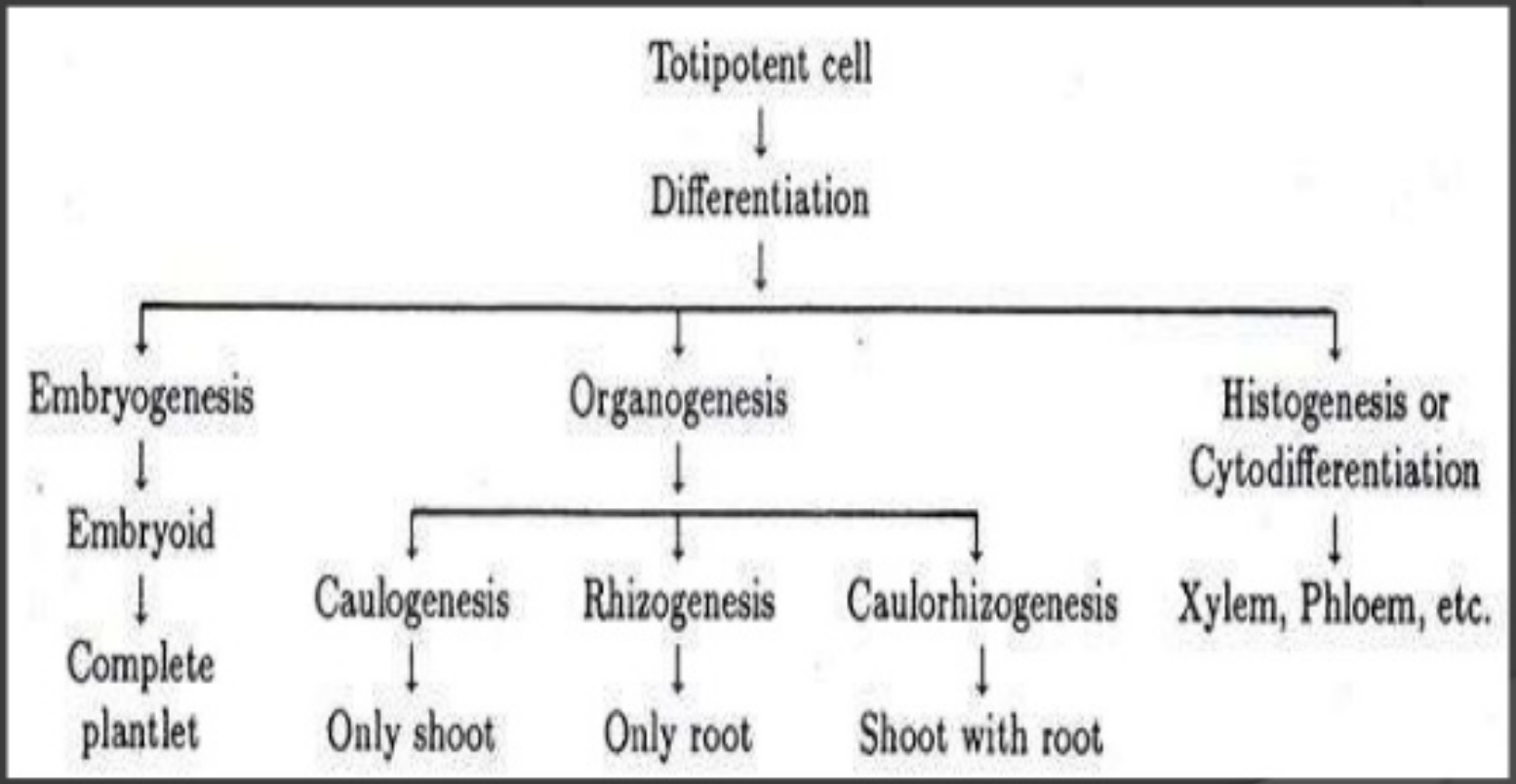
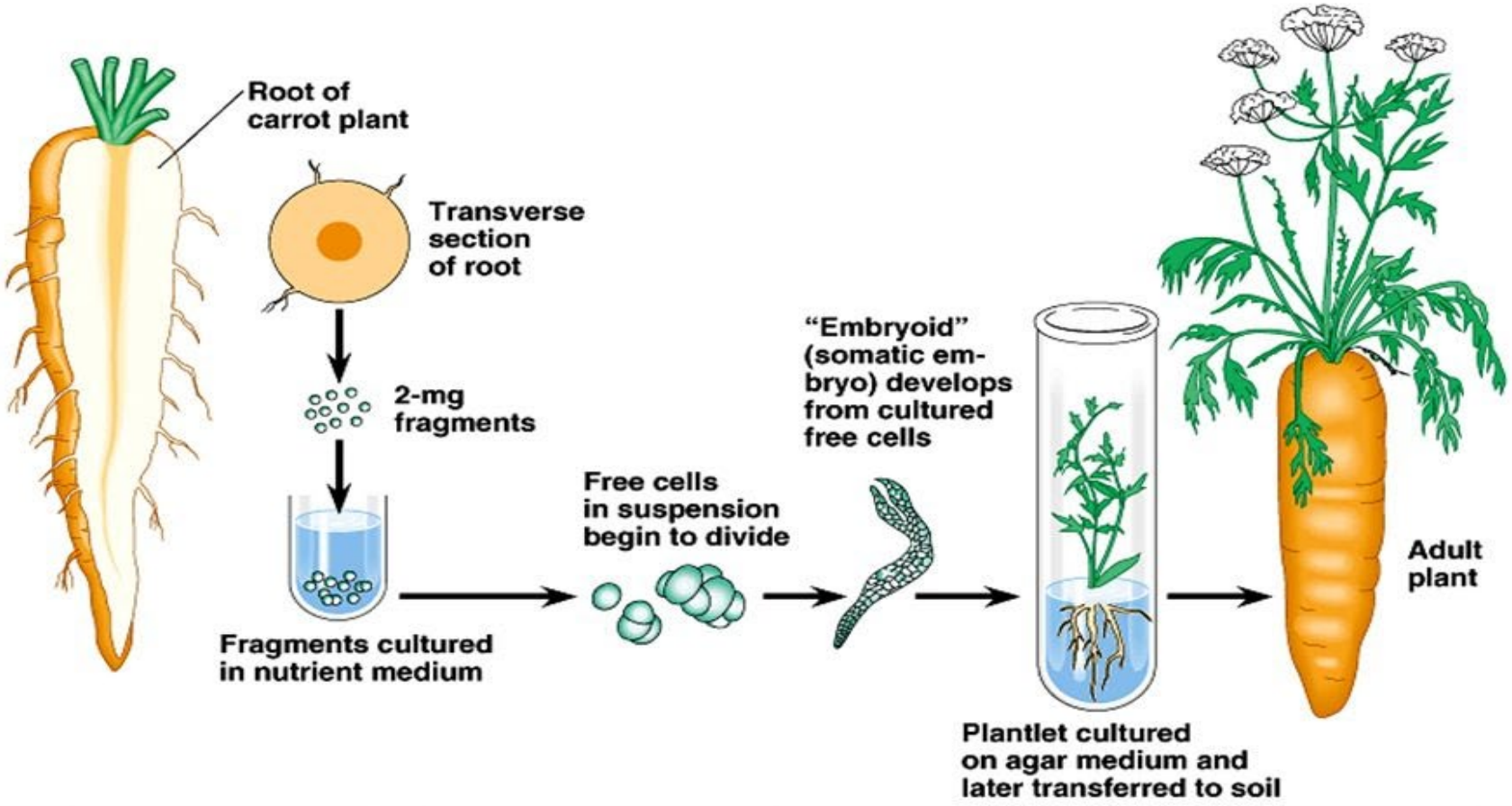


Fig 2 : Differentiation of totipotent plant cell

Expression of Totipotency in culture



In plants, cells remain **totipotent**—
all genes can be activated, and any cell can form any part of the organism

2.2 Basic Technique of PTC

- 1. Media preparation
- 2. Sterlization of Glasswares
- Inoculation of explant
- Incubation Of Explant
- Subculturing
- Hardening

TYPES OF CULTURE MEDIA

- BASED ON THE AGAR CONCENTRATION (Consistency):

  1. *Solid media – 2% agar.*
  2. *Liquid media – absence of agar.*
  3. *Semi solid media – 0.2-0.5% agar*

Major Types of Media:

- **White's medium:**
  This is one of the earliest plant tissue culture media developed for root culture.
- **MS medium:**
  Murashige and Skoog (MS) originally formulated a medium to induce organogenesis, and regeneration of plants in cultured tissues. These days, MS medium is widely used for many types of culture systems.
- **B5 medium:**
  Developed by Gamborg, B5 medium was originally designed for cell suspension and callus cultures. At present with certain modifications, this medium is used for protoplast culture.
- **N6 medium:**
  Chu formulated this medium and it is used for cereal anther culture, besides other tissue cultures.
- **Nitsch's medium:**
  This medium was developed by Nitsch and Nitsch and frequently used for anther cultures. Among the media referred above, **MS medium is most frequently used in plant tissue culture work due to its success with several plant species and culture systems.**

mM	MS	B5	1/2 MS	DKW	WPM	JADS
Macro Elements						
$Ca(NO_{3)2}.4H_2O$						5.000
$Ca(NO_3)_2.2H_2O$				8.300	2.350	
$CaCl_2$	2.990	1.020	1.500	1.010	0.650	
KH_2PO_4	1.250		0.630	1.950	1.250	3.000
K_2SO_4				8.950	5.680	
KNO_3	18.700	24.730	9.400			8.000
$MgSO_4$	1.500	1.010	0.730	3.000	1.500	
$MgSO4.7H_2O$						3.000
$(NH_4)_2SO_4$		1.010				
NaH_2PO_4		1. 0900				
NH_4NO_3	20.610		10.300	17.600	5.000	4.000
Micro Elements						
$CoCl_2.6H_2O$	0.000	0.000	0.000			0.000
$CuSO_4.5H_2O$	0.000	0.000	0.000	0.000	0.000	0.005
$Na_2EDTA.2H_2O$						0.200
$FeSO_4.7H_2O$						0.200
FeNaEDTA	0.100	0.100	0.100	0.120	0.100	
H_3BO_3	0.100	0.048	0.100	0.078	0.100	0.050
KI	0.005	0.005	0.005			
$MnSO_4.H_2O$	0.100	0.059	0.100	0.200	0.130	0.075
$Na_2MoO_4.2H_2O$	0.001	0.001	0.001	0.002	0.001	0.001
$ZnSO_4.7H_2O$	0.030	0.007	0.030	0.072	0.030	0.015
Vitamins						
Glycine	0.0266		0.0266	0.0266	0.0266	0.0266
Myo-Inositol	0.5600	0.5600	0.5600	0.5600	0.5600	0.5600
Nicotinic acid	0.0041	0.0081	0.0041	0.0081	0.0041	0.0041
Pyridoxine HCL	0.0024	0.0049	0.0024		0.0024	0.0024
Thiamine HCL	0.0003	0.0030	0.0003	0.0059	0.0030	0.0003

Media Composition

- 1.Inorganic Nutrients- Minerals & salts are most important for ‘in-vitro’ culture
- 1.Micronutrients- These are required in small quantity i.e. less than 0.5molar/liter. Eg. Salts of Fe,Mn,Zn,I,Cu,Mo,Co
- 2.Macronutrients- These are required in higher quantity i.e. more than 0.5molar/liter. Salts of N,P,K,S,Mg,Na.Ca
- In media Iron is added in Chelate form to dissolve in media & so that Iron can be slowly release & absorbed by plants.eg.Ferric-sodium ethyldiamine tetraacetate(Na2FeEDTA)

Organic Nutrients

Carbohydrates,Vitamines,aminoacids,growth regulators,activated charcoal,solidifying agent

- 1.Vitamines- These are act as catalyst in various metabolic processes.Plant cell in vitro can synthesize vita. But for best growth these can be supplied externally in media.
- Theae are added in the range of 0.1to 10 mg/lit
- Thimine (B10), Nicotin (B3), Folic acid , Ascorbic acd (vit C),Riboflavin (B2),Pyridoxin (B6) ,Ca-Pantothenic acid (B5)

3.Plant Growth regulators/Hormones

- Auxins – Root promoting hormone. Eg.IAA,IBA,2-4-D,NAA
- Cytokinin- Shoot promoting hormone. Eg.Zeatin ,kinetin,6-BPA (benzylamine purine)
- Gibberellins – It stimulate cambial activity , stem elongation eg.Gibberellic acid (GA3)
- * hormones are thermolabile and so can't be autoclave.
- 4.Atibiotic- to control bacterial infection.Kanamycin,streptomycin. Added in less quantity.

- Carbon source:
  - The important carbon source in culture medium is carbohydrates and mainly 2-5% sucrose.
  - Others include maltose and galactose.

- Amino acids:
  - Until unless the nitrogen is supplied through the inorganic source, the addition of amino acids is not necessary.
  - However cysteine is added to prevent the oxidation of phenolics and there by blackening of the tissues.
  - Combination of glutamine and arginine is added to use in protoplast culture.
- Activated charcoal:
  - 0.5 to 3% acid washed activated charcoal is added to the media has both favourable and unfavourable effects.

2. Nutrient Media Composition and Preparation

e. Amino acid:

Serve as a source of nitrogen. Most commonly use aminoacid are L-aspartic acid, L-Aspragin, L-glutanic acid, L-glutamine, L-argine.

f. Solidifying agent:

Most commonly ager (obtain from seaweed) i.e. red alge, *Glidium amansii*.

It do not react with constituent of media and not digested by enzyme. Generally from 0.5 to 1%.

g. pH Effect:

Affect the uptake of ions, optimum 5-6 is required for development of culture tissue. pH should be mention before sterilization of media.

MEDIA PREPARATION PROCEDURE

1. Assemble all chemicals in work area before beginning.
2. Accurately weight the media base calculated “powder” by electronic balance.
3. Add the powder into flask.
4. Add distilled water to the flask, for making the correct volume.
5. Heat & stir (agar will be burn if is not stirred) until all of the ingredients go into solution. When the media boils, it is ready for sterilization.
6. For sterilization, autoclave should be used, flask top should be covered by aluminum foil to prevent contamination.

STERILIZATION TECHNIQUES:

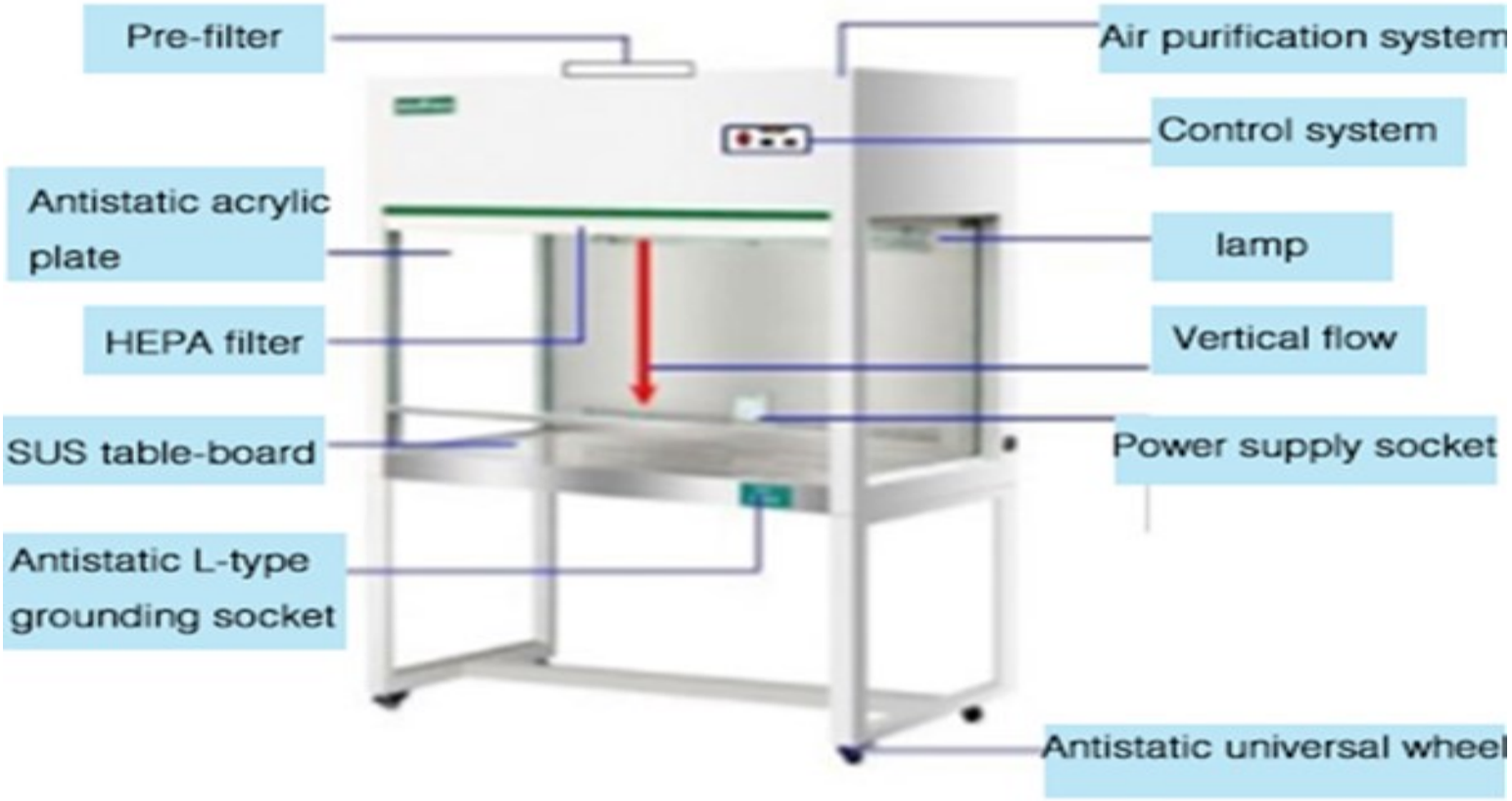
- **Sterilization Methods Used in Tissue Culture Laboratory** - All the materials, e.g., vessels, instruments, medium, plant material, etc., used in culture work must be freed from microbes. This is achieved by one of the following approaches:
  (i) dry heat treatment,
  (ii) flame sterilization,
  (iii) autoclaving,
  (iv) filter sterilization,
  (v) wiping with 70% ethanol, and
  (vi) surface sterilization.


AUTOCLAVE
PYROSIL

Oven

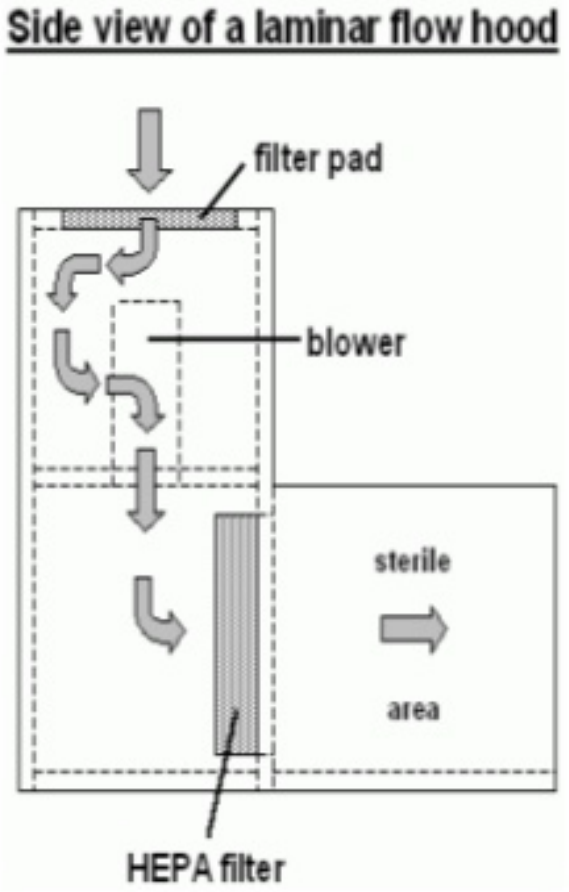
(for dry heat sterilization.Temperature upto 380 degree celsius)

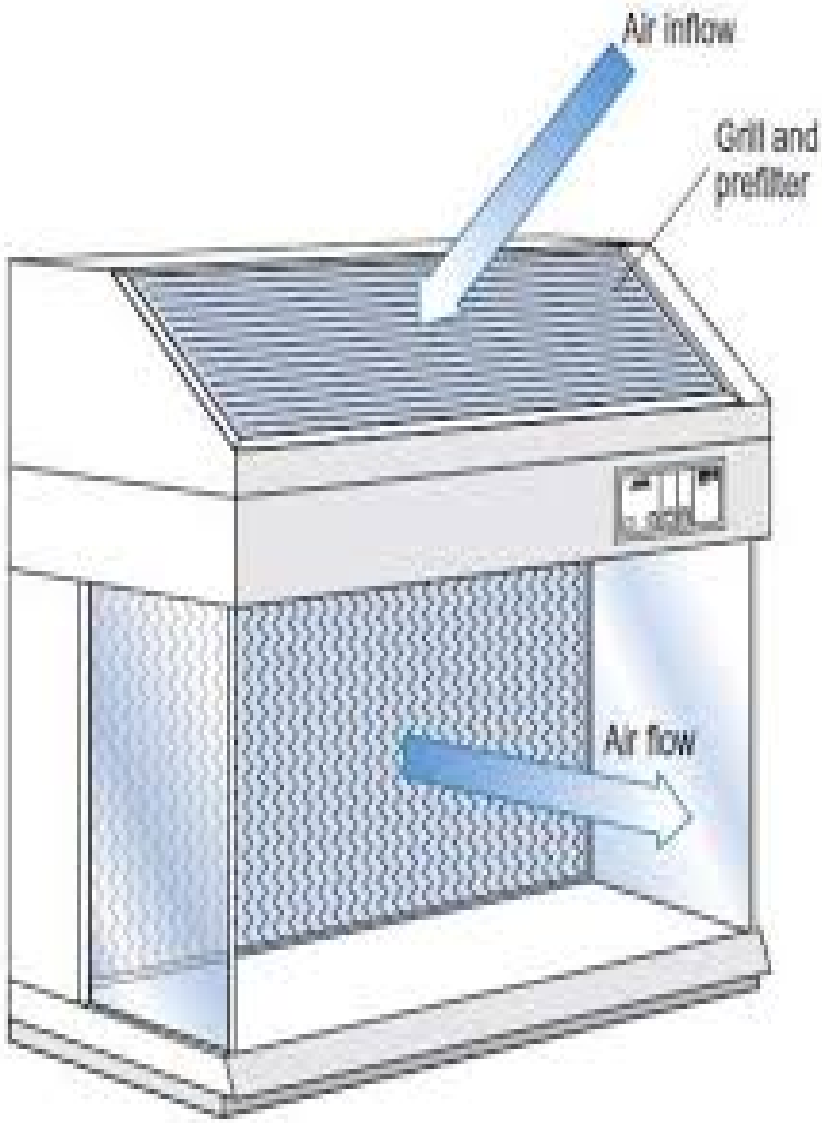
Laminar Air flow
(HEPA FLTERS)



Working Principal

- A laminar flow hood consists of a filter pad, a fan and a HEPA (High Efficiency Particulates Air) filter.
- In a laminar flow hood the air is passed through a HEPA (High Efficiency Particulates Air) filter which removes all airborne contamination to maintain sterile conditions.
- The fan sucks the air through the filter pad where dust is trapped.
- After that the prefiltered air has to pass the HEPA filter where contaminating fungi, bacteria, dust etc are removed.
- Now the sterile air flows into the working area where you can do all your flasking work without risk of contamination.




Air inflow
Grill and prefilter
Air flow

Cleaning of glassware

Borosilicate glassware (Corning/Pyrex) is used. Graduated measuring cylinders, conical flasks beakers petridishes, pipettes (2 ml, 5 ml and 10 ml) glass rods centrifuge tubes culture vials, culture tubes bottles

Physical methods of sterilization

Heat Sterilization

Moist Heat Sterilization

- At temperatures below 100°C
- At a temperature of 100°C
- At temperatures above 100°C

Dry Heat Sterilization

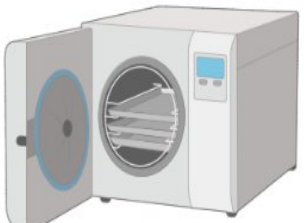
- Red Heat, - Flaming, - Incineration
- Infrared radiation, - Hot air oven

Filtration

- Filtration sterilization of liquids
- Filtration sterilization of gases

Irradiation

- Ultraviolet (non-ionizing) radiation
- Ionizing Radiation



Sound (sonic) waves

Pressure (Pascalization)

Sunlight (Solar Disinfection)

Instruments :-

- Sterilized by dipping in 95% ethanol followed by flaming and cooling
- Glass bead sterilizer and infra-red sterilizer are available commercially operated by electricity these instruments are safer and not a fire hazard
- **Glass bead sterilizer** has glass bead in a heated cavity where a temperature of nearly 250^0C is maintained instruments are pushed into the cavity for 5-7s
- **Infrared sterilizer** also has a cavity where a temperature of nearly 700^0C can be achieved by infra-red wave heating. Exposure of 2-5 s is effective for sterilization of instruments

Glass bead sterilizer

PHYSICAL METHODS:

1. HEAT STERILIZATION:

- Heat sterilization is the most widely used and reliable method of sterilization, involving destruction of enzymes and other essential cell constituents.
- This method of sterilization can be applied only to the THERMO STABLE PRODUCTS, but it can be used for MOISTURE-SENSITIVE MATERIALS.

  i) Dry Heat (160-1800˚C) Sterilization for thermo stable products

  ii) moist heat (121-1340 ˚C) sterilization is used for moisture- resistant materials.

Glassware, plasticware and Medium sterilization:-

a) Dry heat

- Used for glassware, metal instruments etc. dry-heating in an oven at 160-180^{0}C for 3 hours approx. (1 hr heat up period to reach to temp and cooling period)

- Wrapping in aluminum foil

- Cannot be used for plastic ware, however, certain plastic wares can also be heat sterilized (Instructions of the manufacturer must be read before doing this)

Media room

- Maintain cleanliness, removal of contaminated culture, restricted entry

Washing room

- Contaminated cultured should be autoclaved and discarded with utmost care, maintain general cleanliness

Transfer area

- A sterile area is required for performing various aseptic manipulations during tissue culture. This ensures that contaminants do not gain entry into the culture vial

- Laminar air flow cabinets of various shapes and sizes are available commercially. These cabinets allows the tissue culturists to work in the sterile environment for long stretch of time. It provides a covered enclosed area for working

Transfer area

- There are UV lights inside the chambers which are switched on for 10-15 minutes before using the laminar air flow cabinet
- It has small motors at the base for blowing the air which is first passed through coarse filter. This step ensures removal of large contaminants. Then the air passes through fine filters called HEPA filters. HEPA stands for 'High Efficiency Particulate Air' . These filters removes impurities which are larger than 0.3 µm therefore the air coming out of these is clean.
- Air coming out of these filter comes with some force which prevent entry of contaminants from the worker or environment into the working area
- A gas burner or spirit lamp facility is also available for flaming the instruments

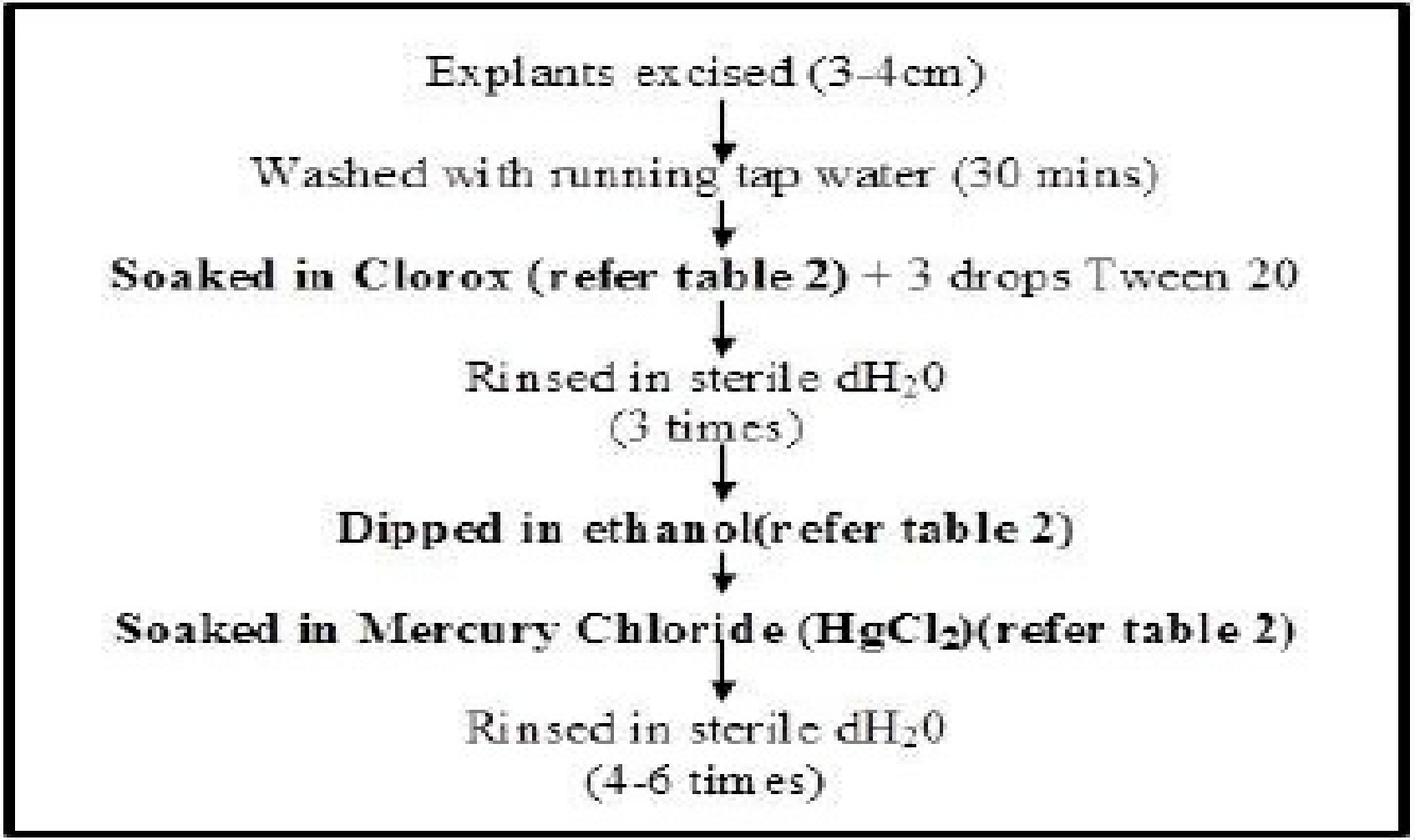
Growth room

- Maintain cleanliness, removal of contaminated culture, restricted entry

Surface sterilization of explant

- Explant is small plant part which is going to developed into new plantlet in-vitro in tissue culture laboratory.
- Example of Explant – It may be single cell , protoplast (cell without cell wall) , tissue, oran , pollen grain, Embryo , seed etc.
- Before inoculation explant should be sterilizied by chemicals like mercuric chloride , bromine water , alcohol etc.So that microorganism present on it will kill down & explant will free from microbes.

Surface sterilization of explant



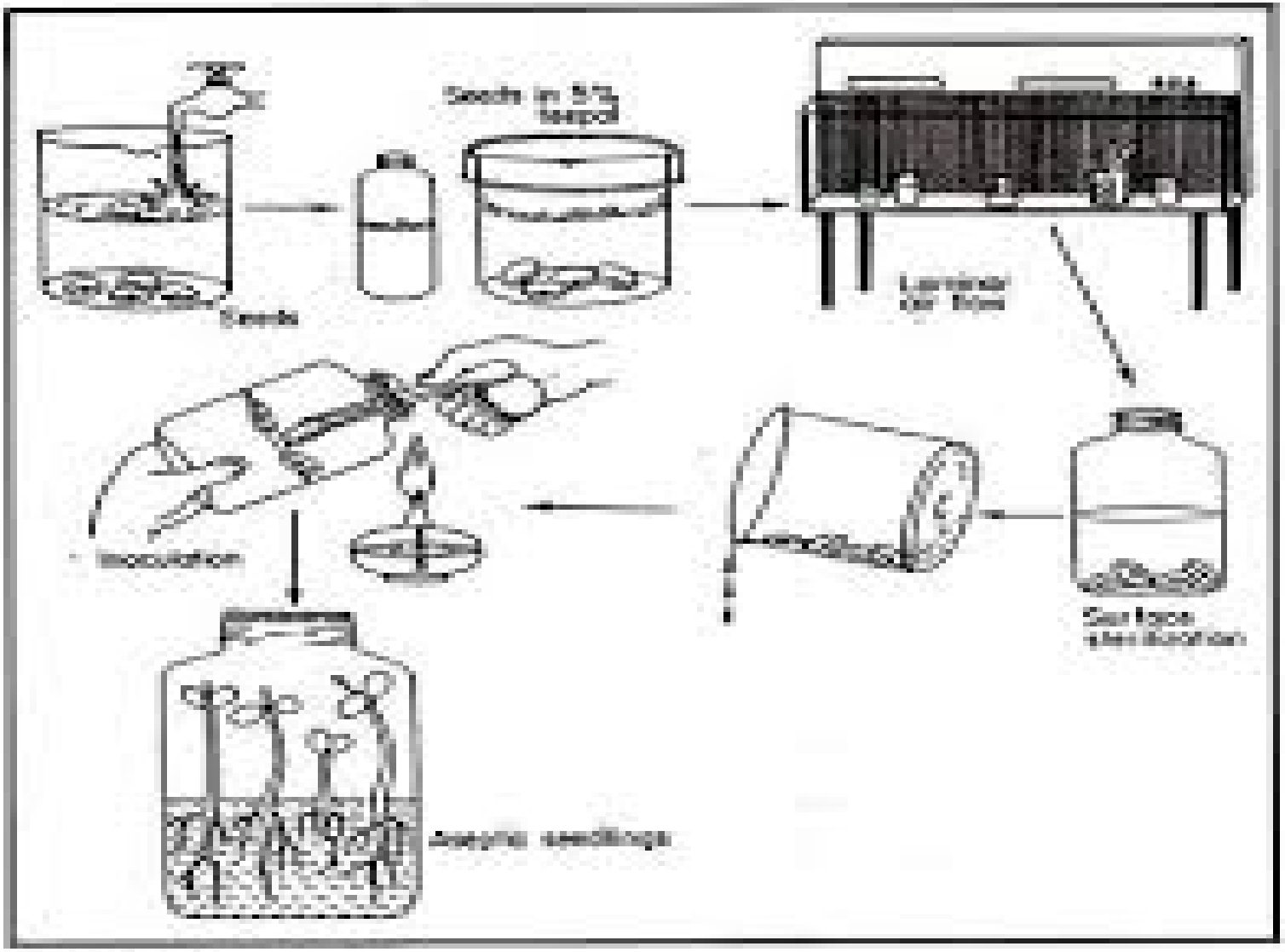


Fig 1.11

Flow diagram illustrating the preparation of aseptic plants from seeds

Inoculation

- Defination – Placing of plant explant (small part of leaf ,root,etc or embryo , anther etc.) on the nutrient medium under Laminar Air Flow is called inoculation.
- Inoculation is carried out under Laminar Air Flow or in between two burners also.

Inoculation of Explant

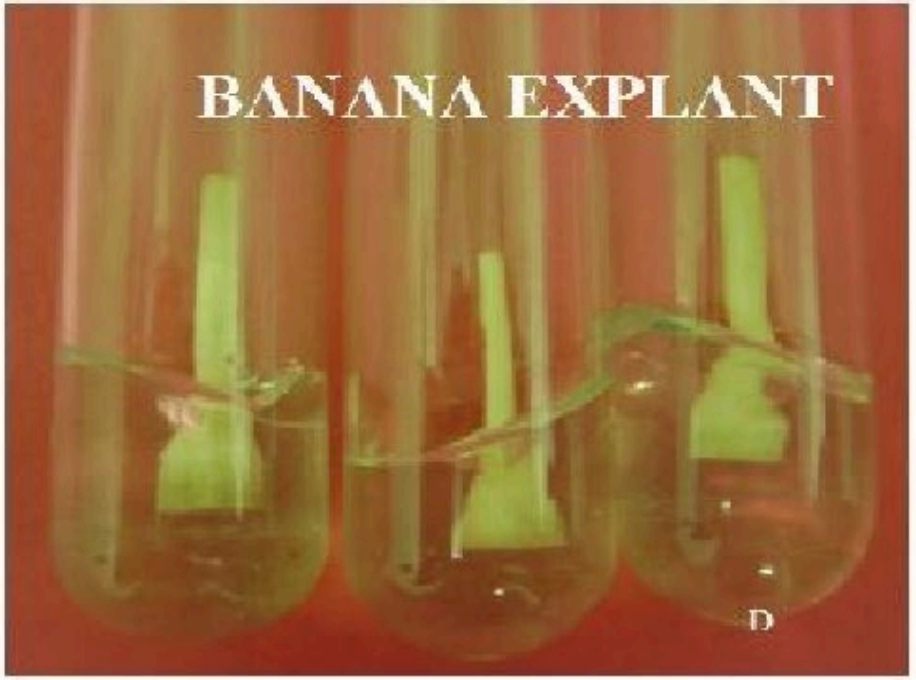
- Swab the working table with alcohol.
- Put all sterilized required glasswares, instrument on laminar air flow cabinet.
- Put on UV lamp on for one hour.then switch off
- Wear clean apron,mask at the time of inoculation
- Deep needle,pointer,scalpel,forcep etc.in alcohol.
- Light the spirit lamp.
- remove the cotton plug of culture tube with same finger & Flame the neck of culture tube to kill microbes.
- Take forcep & flame it.Take explant with it & insert in MS medium of culture tube .

Inoculation of explant under laminar air flow


Banana suckers
A


Sterile shoot tip
B


MS MEDIUM
C


BANANA EXPLANT
D

Incubation of explant

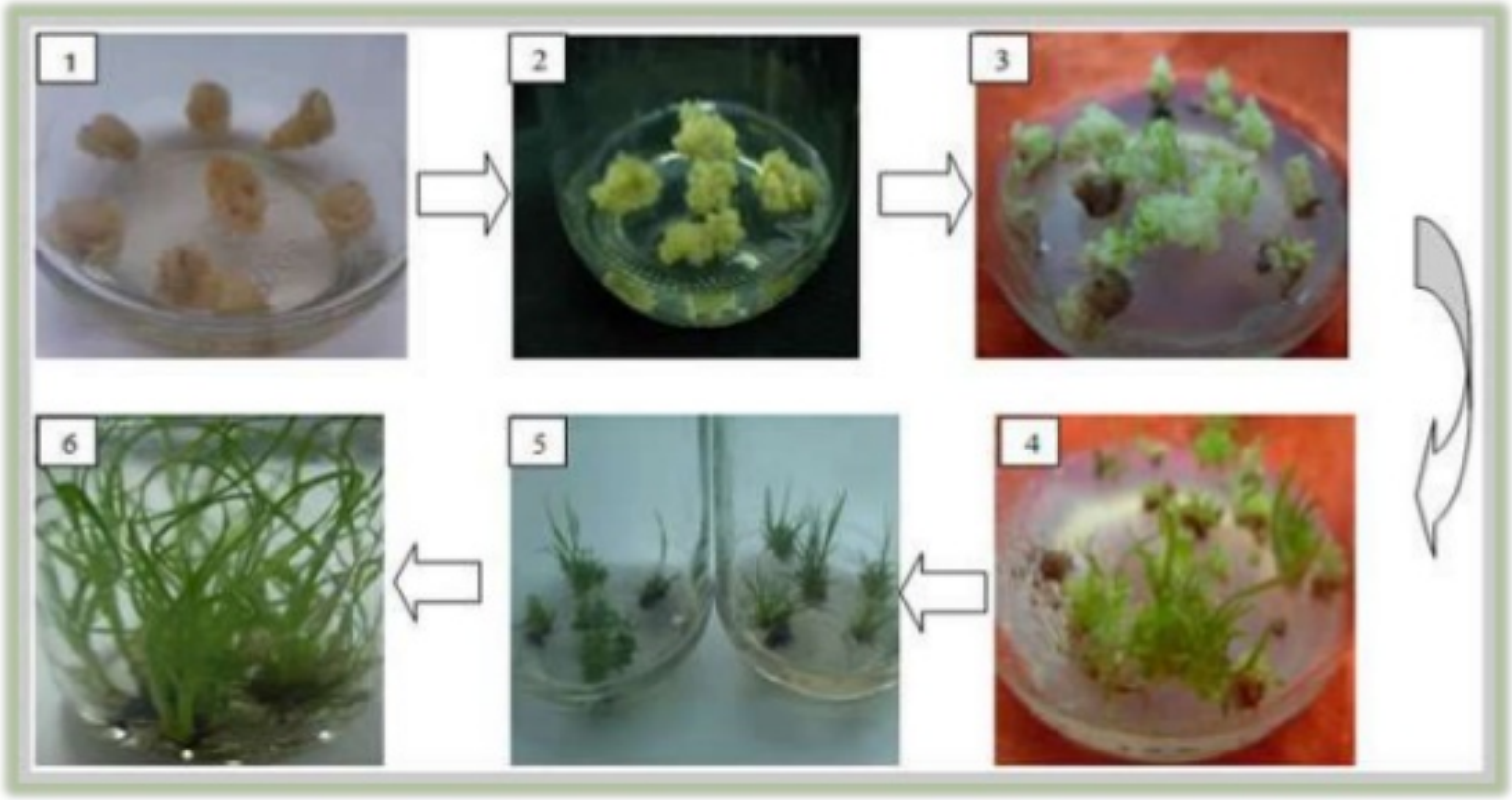
- Inoculated tubes/jars are keep in incubation room.
- Temp -25-28 Oc ,humidity 85 to 90 %
- Light intensity-4 to 10* 103 lux for 16 hrs

Incubation:

- The culture vessels with inoculated explants are incubated under controlled conditions of temperature, illumination and humidity.
- The cultures are incubated for 3 to 4 weeks during which, the cells of the explant absorb the nutrients, grow and undergo repeated divisions to produce a proliferating undifferentiated mass of cells known as **callus** or produces shoots or roots directly.
- The explant or callus cultured on different combinations of auxins and cytokinins will produce shoots or roots, called as **organogenesis**.
- High proportion of auxin and low proportion of cytokinin induce root development from callus called and is known as **rhizogenesis**.
- Low proportion of auxin and high proportion of cytokinin in the medium induce shoot development from the callus and is called as **caulogenesis**.

Growth Room

Fig 2 Stages of callus



Callus induction and proliferation (1-2); Differentiation/(regeneration of shoots (3-4), and the establishment of plantlet rooting induction (5-6).

Fig 1 Stage of shoot tip culture

Sugarcane epical bud used as Explant

Establishment of shoot tip

shoot Initiation in MS

Shoot Elongation

Initiation of multiple shooting

shoot multiplication

Rooting

Hardening

Hardening

- Healthy/elite plantlets are exposed to the natural conditions in a step wise manner.
- It is a gradual acclimatization of *in vitro* grown plants to *in vivo* condition.
- The plantlets are transferred to the pots/polyghene bag and immediately irrigated with inorganic/nutrient solution.
- Plants are kept in the hardening room where controlled conditions of light, humidity and temperature are maintained.
- Plants are maintained under high humidity for 10-20 days and subsequently transferred in the field so as to grow under natural conditions. The success rate of micropropagation depends on the survival of the plantlets when transferred from culture to the soil (field).

- Primary hardening –plants are removed from nutrient medium from tissue culture bottles. Then washing of roots with water.ang again replant or transfer it to plastic pot filled with liquid nutrient medium.kept it n green house for 6-8 week
- Secondary hardening- The above plants are transferred to poly bags filled with potting mixture of saw dust + soil + coconut fibres .It is also called as Cocopit.keep these for 6-8 weeks under green house.

Hardening

- Once the new plant becomes large enough to handle, it can be removed from the culture tube and planted in a growing medium. Newly transplanted plantlets are placed in controlled conditions of light, temperature and humidity for several weeks as they adjust to their new environment.
- This is necessary as many young tissue culture plants have no waxy cuticle to prevent water loss.

Primary Hardening- keep plants in liquid nutrient medium
Secondary Hardening- plants grown in potting mixture. Soil + saw dust

SECONDARY HARDENING