PLANT TISSUE CULTURE

(PART 1)

BSc BOTANY
CORE COURSE CODE: BOT5B08T
SEM V

By Dr. Simmi PS

DEPARTMENT OF BOTANY
ST MARY'S COLLEGE, SULTHAN BATHERY

TISSUE CULTURE Module-1 (12 hrs)

1. Plant tissue culture – Principles and techniques; Cellular totipotency; in vitro differentiation – de differentiation and re-differentiation. (2 hrs)
2. Tissue culture medium – Basic components in tissue culture medium – Solid and liquid medium; Murashige and Skoog medium – composition and preparation.
3. Aseptic techniques in in vitro culture – sterilization – different methods – sterilization of instruments and glassware, medium, explants; working principle of laminar air flow and autoclave. (2 hrs)
4. Preparation of explants– surface sterilization, inoculation, incubation, subculturing. (2 hrs)
5. Micropropagation - Different methods – apical, axillary bud proliferation, direct and indirect organogenesis and somatic embryogenesis. (2 hrs)
6. Different phases of micropropagation – multiple shoot induction, shoot elongation, in vitro and in vivo rooting hardening, transplantation and field evaluation; advantages and disadvantages of micropropagation, somaclonal variation. (2 hrs)

1. **Plant tissue culture – Principles and techniques; Cellular totipotency; in vitro differentiation – de differentiation and re-differentiation.**

INTRODUCTION

WHAT IS PLANT TISSUE CULTURE?

- **Plant Tissue culture is the technique of '*In vitro*' culture of plant cells, tissues or organ on nutrient medium under aseptic conditions usually in a glass container.**

- **Tissue culture produces clones, in which all product cells have the same genotype (unless affected by mutation during culture). It has applications in research and commerce**

FATHER OF PLANT TISSUE CULTURE

- In 1902, a German Botanist Gottlieb Haberlandt developed the concept of culture of isolated cells of Tradescantia in artificial condition. Though his experiment failed to induce the cells to divide.

- The theoretical basis for plant tissue culture was proposed by Gottlieb Haberlandt in his address to the German Academy of Science in 1902 on his experiments on the culture of single cells

Gottlieb Haberlandt

PRINCIPLES OF PLANT TISSUE CULTURE

1. PLASTICITY:

Plasticity is the ability of the plants to endure extreme conditions and predation and to modify their growth and development in accordance with environmental conditions.

- **It is the adaptability and endurance of plants in response to changes in environmental conditions.**

- **Plasticity enables plants to modify their metabolism, growth and development as adaptations to their environment.**

- **As far as plant tissue culture and regeneration is concerned, plasticity is significant as it enables all tissues to initiate cell division or undergo development in response to stimuli.**

2. TOTIPOTENCY:

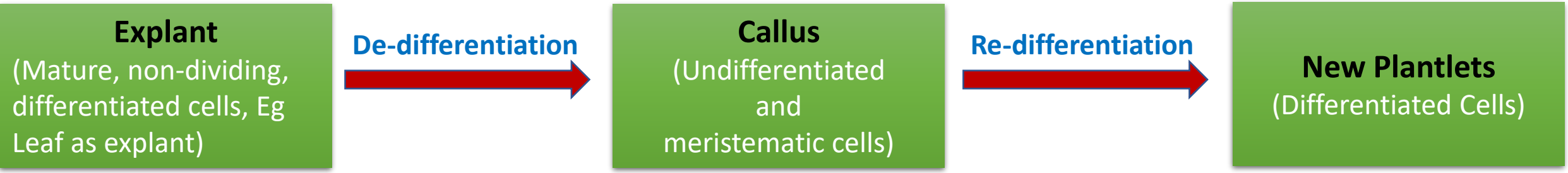
The basis of plant cell and tissue culture is totipotency. The term totipotency was coined by Morgon (1901).

In general terms, totipotency is the genetic potentiality or property of a cell to produce a whole organism or whole parent plant in the presence of correct physical and chemical stimulus.

Totipotency is defined as the ability of an isolated, fully differentiated and mature plant cell to revert or dedifferentiate to a meristematic state and then to divide, re-differentiate and develop to a whole plant.

All cells are not totipotent.
Cells that lack cytoplasm and nucleus are not totipotent.
Eg: tracheids, fibers, sclereids, vessel elements

The two inherent phenomenon that are involved in cellular totipotency are:
a) Dedifferentiation
b) Re-differentiation



a. DEDIFFERENTIATION :

When an explant from differentiated tissue is used for culture on a nutrient medium, the non- dividing quiescent cells first undergo certain changes to achieve a meristematic state. The phenomenon of the reversion of mature cells to the meristematic state leading to the formation of callus is called dedifferentiation.

Callus is a disorganised proliferated mass of parenchyma. Callus formation is governed by the source of explant, nutritional composition of medium and environmental factors.

b. REDIFFERENTIATION:

The component cells of callus have the ability to form a whole plant, a phenomenon described as “redifferentiation”.

Anyone who wishes to start plant tissue cultures should have in mind the following basic principles:

(1)select an appropriate explant from a healthy and vigorous plant,

(2) eliminate microbial contamination from the surface of the explant,

(3) inoculate the explant in an adequate culture medium, and

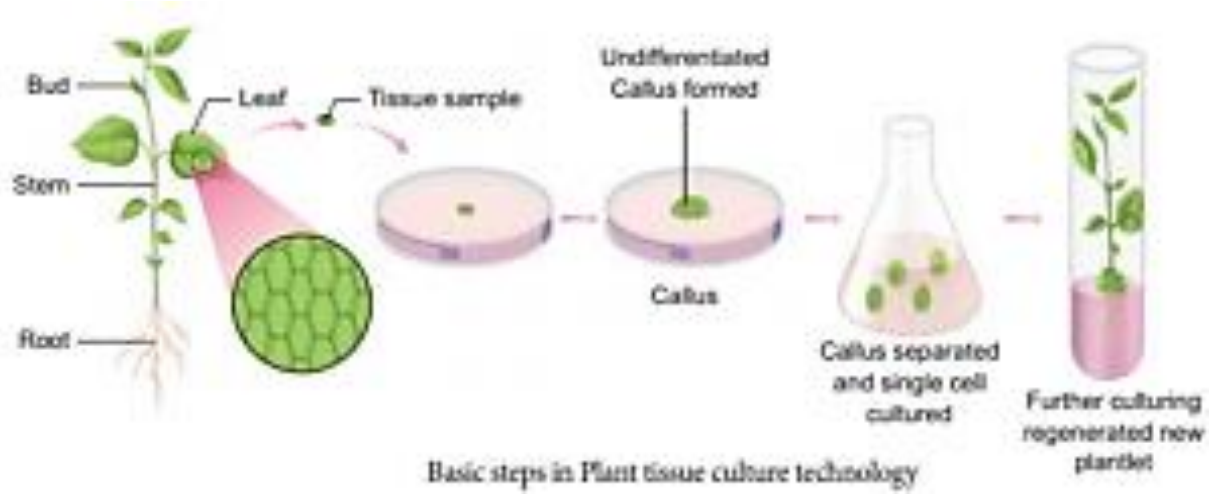
(4) provide the explant in culture with the proper controlled environmental conditions.

In the case of in vitro regenerated plants, they are subjected to an adaptation process (acclimatization) in the greenhouse before the transference to *ex vitro* conditions.

Techniques used for plant tissue culture *in vitro*

The major steps used in *in vitro* culturing of an explant are:

1. **Surface sterilization of the explant**
2. **Preparation of the Medium**
3. **Inoculation**
4. **Callus Growth**
5. **Subculturing**
6. **Organogenesis**
7. **Direct regeneration**
8. **Acclimatisation and transfer to the field**



Basic steps in Plant tissue culture technology

1. **Surface sterilization of the explant:**

Explant: The plant part to be cultured *in vitro* is known as Explant.

Development of a tissue is the result of cell division, cell elongation and cell differentiation.

Considerations for explant

- **explant are taken from young healthy plants.**
- **Presence of parenchyma in the tissues as parenchyma responds faster to tissue culture conditions**
- **Size: usually 2-5 mm.**
- **Sterilization of explant is carried out by chemical sterilants like 1-2% solution of sodium or calcium hypochlorite or 0.1% mercuric chloride.**
- **All procedures are carried under aseptic condition.**

2. Preparation of the Medium

Appropriate tissue culture medium is prepared in advance to collection of explant. The medium prepared may be solidified using agar (6-8 gm/l) or may be used as liquid medium.

3.Inoculation

Inoculation is the transfer of explants to culture vials. The procedure is carried out in the inoculation chamber/laminar air flow under aseptic conditions .

4.Callus Growth:

- Callus tissue is defined as unorganized, proliferative mass of cells produced from isolated plant cells, tissues or organs when grown aseptically on artificial medium under controlled experimental conditions.

- Callus formation is controlled by

  - Source of the explant:
  - Medium composition: nutrient medium (with appropriate auxin and cytokinin combination)
  - Environmental factors: temperature (25-28^{0}C), light duration (certain plant need complete dark and certain a photoperiod of 16hrs light and 8 hrs dark or 12 hrs of light and dark), and light intensity, humidity.

  Under optimal culture condition the surface of the explants get covered with by amorphous (without clearly defined shape or form) mass of loosely arranged thin walled cells. This mass of cells is called callus.

  Callus is characterized by abnormal growth and has potential to produce roots, shoots and embryoids.

5. Subculturing:

Callus has to be subcultured to fresh medium periodically to avoid nutrient and water depletion and accumulation of toxic metabolites.

6.Organogenesis:

Occurs under the influence of growth regulators. Occurs in two stages:

1. Caulogenesis or shoot initiation : low auxin to cytokinin
2. Rhizogenesis or root initiation: high auxin to cytokinin

The formation of an embryoid from the callus is called **Somatic Embryogenesis**.
Optimal hormonal condition can lead to formation of entire plantlet. This process is called **Regeneration.**

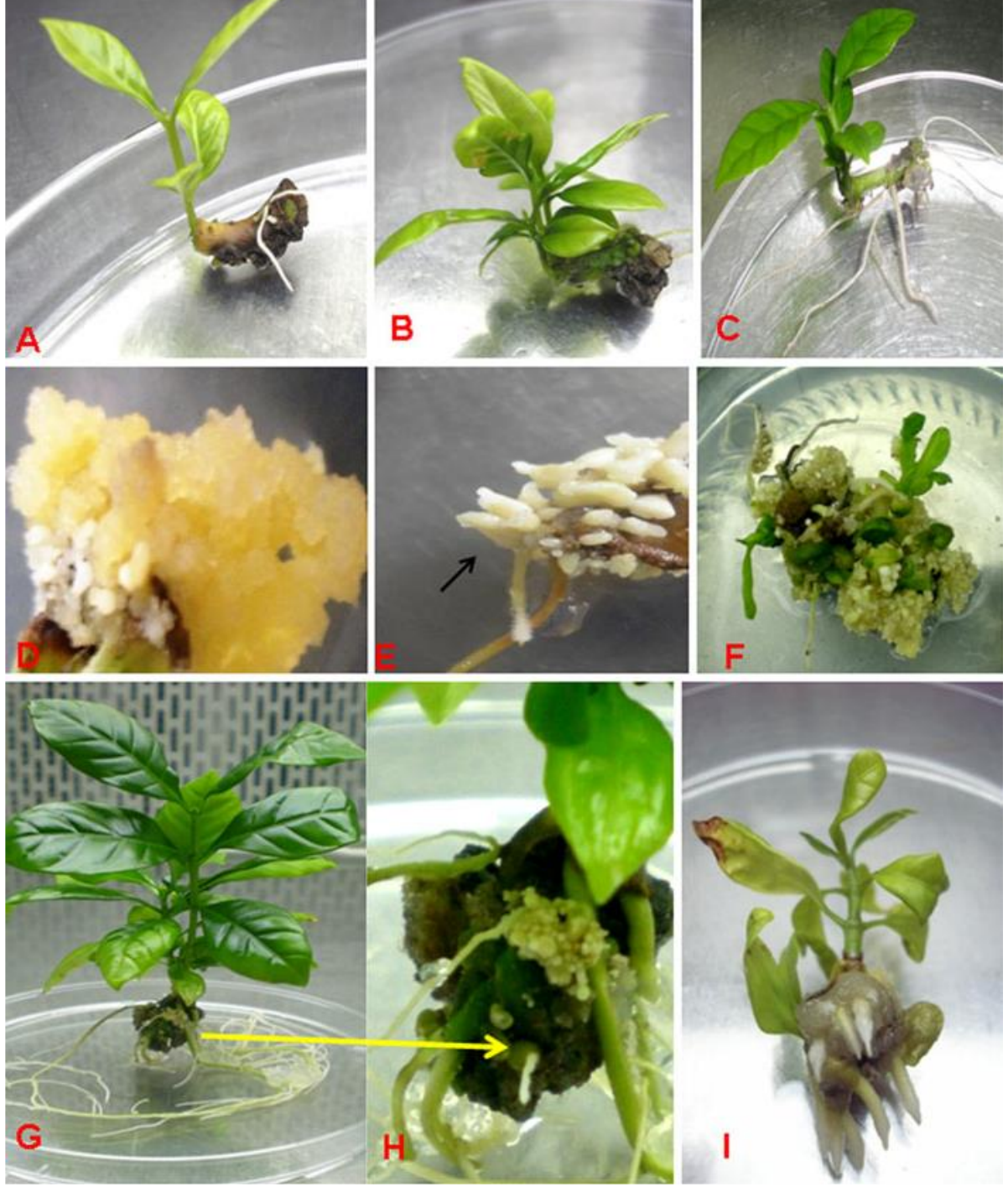
7. Direct regeneration:

Plantlets can grow direct from the calli. And this process is known as direct regeneration.

8.Acclimatisation and transfer to the field

Last stage is process to make rooted plants adjust to the field conditions and this stage is know as Acclimatization.

- Plants are taken out from the medium, washed thoroughly in running water to remove agar.
- The plants are then put in low mineral salt medium (LMSM) for 28-48 hrs.
- The plants are transferred to pots containing autoclave sterilized mixture of clay, sand and vermicompost (1:1:1).
- The pot is covered with transparent polythene to maintain humidity and kept undisturbed for 15-30 days.
- Post this acclimatization, the plants are transferred to the field.



Coffee tissue culture stages

Applications of plant tissue culture

Plant tissue culture is used widely in the plant sciences, forestry, and in horticulture. Applications include:

• For Micropropagation: The commercial production of plants used as potting, landscape, and florist subjects, which uses meristem and shoot culture to produce large numbers of identical individuals.

•To conserve rare or endangered plant species.

•Large-scale growth of plant cells in liquid culture in bioreactors for production of valuable compounds, like plant-derived secondary metabolites and recombinant proteins used as biopharmaceuticals.

•To cross distantly related species by protoplast fusion and regeneration of the novel hybrid.

•To rapidly study the molecular basis for physiological, biochemical, and reproductive mechanisms in plants, for example in vitro selection for stress tolerant plants.[9]

•For chromosome doubling and induction of polyploidy, for example doubled haploids, tetraploids, and other forms of polyploids. This is usually achieved by application of antimitotic agents such as colchicine or oryzalin.

•As a tissue for transformation, followed by either short-term testing of genetic constructs or regeneration of transgenic plants.

•Certain techniques such as meristem tip culture can be used to produce disease free plants

•Large scale production of artificial seeds through somatic embryogenesis

ADVANTAGES AND DISADVANTAGES OF PLANT TISSUE CULTURE

1. The new plantlets grow in a shorter time.
2. The amount of initial plant material are required very small.
3. The new plantlets are mostly disease free.
4.. The process is independent of seasonal variation and can be done throughtout the year.
5. People who are at cultivating challenging plants like orchids have more success with tissue culture than traditional soil.

1. Requires higher skilled labour and costs more money
2. The plants may be less resilient to diseases due to the controlled type of environment they are grown in.
3. Some tissue cultured plants may show abnormal plant growth

Factors affecting Plant Tissue culture:

- Growth Media
- Environmental factors
- Genetic Factors
- Explant Source

Chapter 2

Tissue culture medium – Basic components in tissue culture medium – Solid and liquid medium; Murashige and Skoog medium – composition and preparation.

WHAT IS IN A PLANT TISSUE CULTURE MEDIUM??

- For suitable and desirable growth of excised plant tissues and organs *in vitro*, suitable artificially prepared nutrient medium is an essential component and this medium is known as a **Culture Medium**.

- A medium containing only **'chemically-defined'** compounds is referred to as a 'synthetic medium' and

- Media used in plant tissue culture contain nutritional components, which are essential for the growth and development of the cultured tissue.

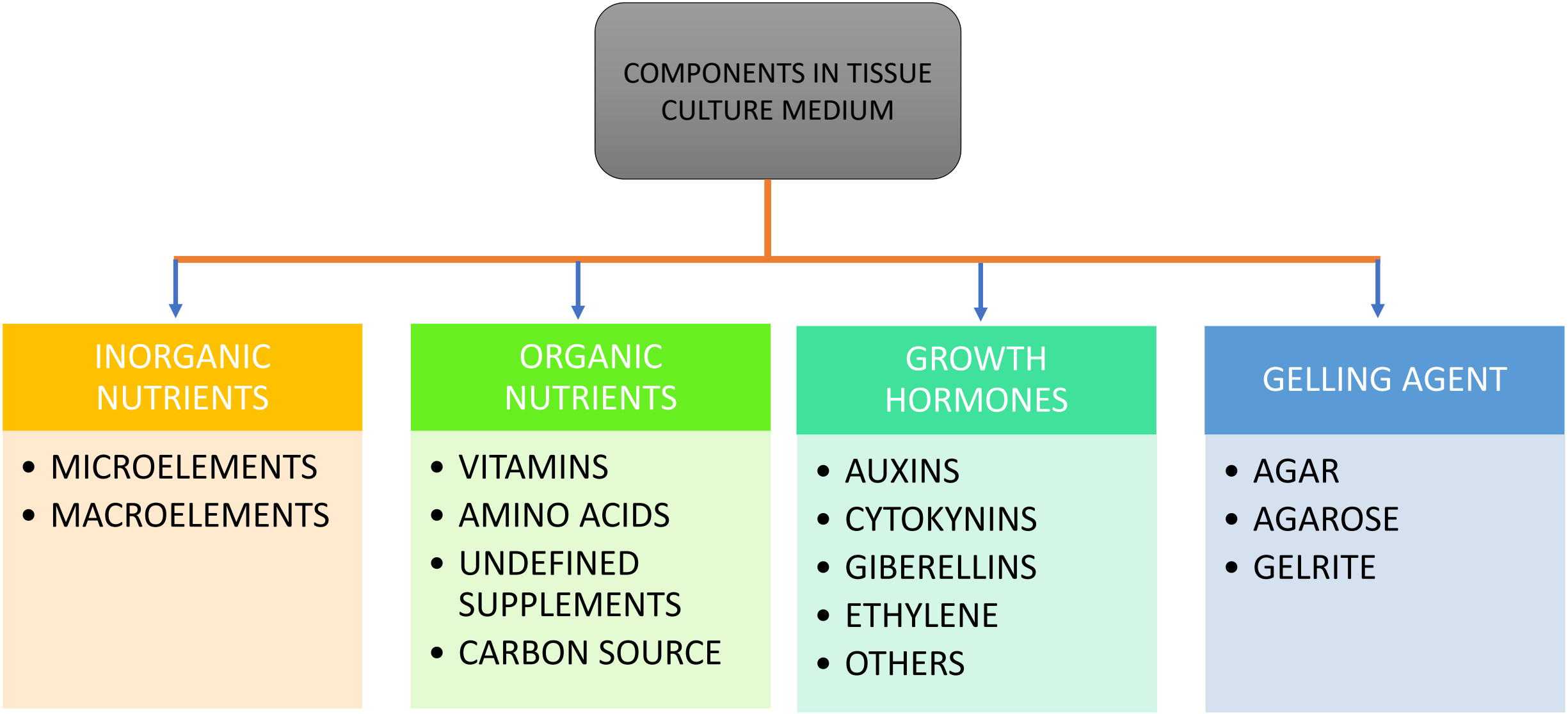
- The success of the tissue culture depends very much on the types of culture media used. No single medium can be suggested as being entirely satisfactory for all types of plant tissues and organs.

Example of some already established Plant Tissue Culture Medium

- Murashige and Skoog (1962): MS – media contain desired salt concentration and widely used
- Linsmaier and Skoog (1965): LS – media contain desired salt concentration and widely used
- White's medium (1963): media contain low salt concentration and used for root culture.
- Gamborg et al.(1968): B5 - media contain much greater proportion of Ammonium and Nitrate ions and used for cell suspention or callus culture.

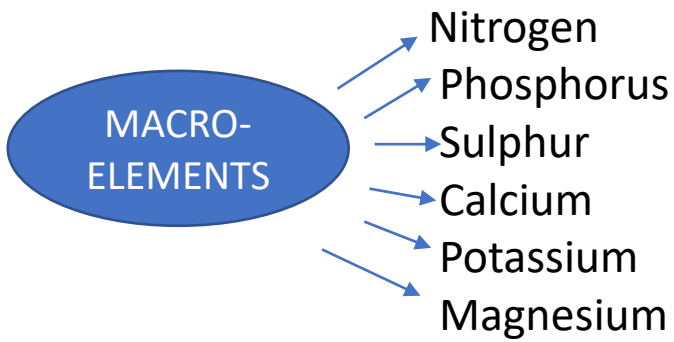
- According to the International Association for Plant Physiology, mmol 1^{-1} should be used for expressing the concentration of macronutrients and organic nutrients and µmol 1^{-1} for micronutrients, hormones, vitamins and other organic constituents in the plant tissue culture medium.

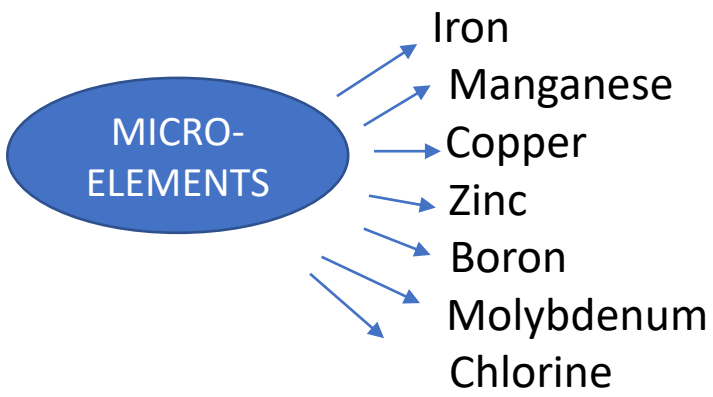
- When mineral salts are dissolved in water they undergo dissociation and ionization. The active factor in the medium is the ions of different types rather than the compounds. One type of ion may be contributed by more than one salt. For example, in Murashige and Skoog's (1962) medium (MS) NO_3- ions are contributed by NH_4NO_3 as well as KNO_3, and K^+ ions are contributed by KNO_3 and KH_2PO_4.


COMPONENTS IN TISSUE CULTURE MEDIUM
INORGANIC NUTRIENTS
• MICROELEMENTS
• MACROELEMENTS
ORGANIC NUTRIENTS
• VITAMINS
• AMINO ACIDS
• UNDEFINED SUPPLEMENTS
• CARBON SOURCE
GROWTH HORMONES
• AUXINS
• CYTOKYNINS
• GIBERELLINS
• ETHYLENE
• OTHERS
GELLING AGENT
• AGAR
• AGAROSE
• GELRITE

INORGANIC NUTRIENTS

- Mineral elements are very important in the life of a plant.
- Essentially, all the elements that are important for whole plant growth in soil are also necessary for plant growth in tissue culture
- Besides C, H, and O, other elements known to be essential for plant growth are: nitrogen, phosphorus, sulphur, calcium, potassium, magnesium, iron, manganese, copper, zinc, boron, molybdenum and chlorine.
- According to the recommendations of the International **Association for Plant Physiology** the elements required by plants in concentrations **greater than 0.5 mmol/L (millimole per liter)** are referred to as **MACROELEMENTS** and those in concentrations less than **0.5 mmol/L** are **MICROELEMENTS**





MACROELEMENTS concentrations >0.5 mmol/L

1. **Nitrogen** : Either Nitrate (NO_3^-) and Ammonium (NH_4^+)
   KNO_3 , NH_4NO_3 , $Ca(NO_3)_2$

- **Contributes to the growth of plants *in vitro* and *in vivo***
- **Is a constituent of the amino acids, proteins, certain hormones and chlorophyll.**
- **An indirect effect of nitrogen on tissue growth is through its influence on the pH of the medium. The form of nitrogen, as NH_4^+or NO_3^-, has a dramatic influence on the morphogenic response of plant tissues *in vitro***
- ***Eg:* Development of anthocyanin *in vitro* has been attributed to deficiency of NO_3^- ions**

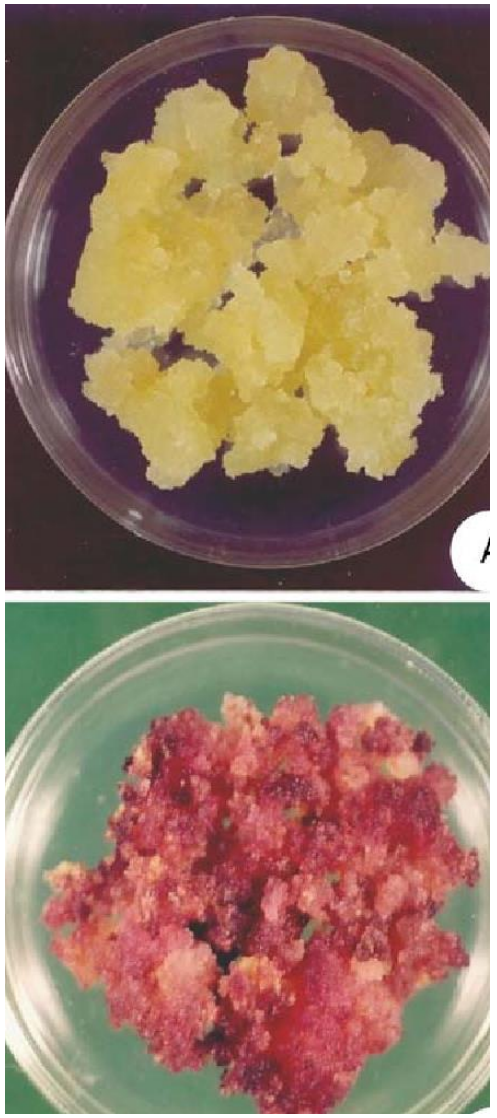


Fig: *Daucus carota* Calli showing anthocyanin pigment

2. Phosphorus as $PO4^{3-}$,K_2HPO_4 or KH_2PO_4 or Na Salts

- **vital for cell division as well as in storage and transfer of energy in plants. Too little phosphorus causes plants to be abnormal and sickly**

3.Potassium as K^+ , KCl or K_2HPO_4

- **necessary for normal cell division, for synthesis of proteins, chlorophyll, and for nitrate reduction.**

4.Sulphur as $SO4^{2-}$ Many SO_4

- **present in some proteins. It is quite often present as an impurity in agar**

5.Calcium as Ca^{2+}, $CaCl_2$ or $Ca(NO_3)_2$

- **as calcium pectate is an integral part of the walls of plant cells and helps maintain integrity of the membrane.**

6.Magnesium Mg^{2+}as $MgSO_4$

- **a component of chlorophyll and a co-factor for many enzyme reactions.**

MICROELEMENTS concentrations <0.5 mmol.l^{-1}

The microelements are essential as catalysts for many biochemical reactions. Microelement deficiency symptoms include reduced lignification (Cu, Fe), rosetting (Zn, Mn), leaf chlorosis (Fe, Zn, Mn) and shoot tip necrosis (B). Certain elements, such as Co and Ni, can inhibit ethylene synthesis.

ORGANIC NUTRIENTS

- VITAMINS :
- Vitamins regulate the metabolic activities of the cells and are required in very minor quantities in tissue culture.
- **thiamine (vitamin B1)** is used for all types of tissue culture. Thiamine is involved in the direct biosynthesis of certain amino acids and is an essential co-factor in carbohydrate metabolism.
- **niacin (vitamin B3), riboflavin (vitamin B2), pyridoxine (vitamin B6), pantothenic acid, vitamin C, vitamin D and vitamin E** are other vitamins that find specific uses in tissue culture.
- Vitamin E is an anti-oxidant ; vitamin C is useful to prevent blackening during explant isolation. Vitamin D has a growth regulatory effect on plant tissue cultures. Riboflavin has been found to inhibit callus formation and improve growth and quality of shoots

AMINO ACIDS

- Amino acids may be directly utilized by the plant cells to stimulate cell growth or may serve as a nitrogen source.
- The most commonly used amino acid is **glycine**. Apart from **these Asparagin, Aspartic Acid, Glutamic acid , Glutamine, Aginine , Cysteine** are also used.

UNDEFINED SUPPLEMENTS

- Numerous complex nutritive mixtures of undefined composition, like **casein hydrolysate (CH), coconut milk (CM), corn milk, malt extract (ME), tomato juice (TJ), and yeast extract (YE),** have also been used to promote the growth of certain calli and organs.

CARBON SOURCE

- In tissue culture, plant cells are not autotrophic and therefore they require external source of carbon and energy.
- The most commonly used carbon source is **Sucrose,** at a concentration of **2% - 3% (20-30 gm/l).**
- Other sources of carbon used are maltose, galactose, mannose, lactose etc.

GROWTH HORMONES

- Along with nutrients, it is generally necessary to add one or more growth hormones to support good growth of tissues and organs.
- The important growth hormones used in tissue culture are : **cytokinins, auxins, and gibberellins (CAG)**

- **1. CYTOKININS**
- In tissue culture media, cytokinins are incorporated mainly for **cell division**, **cell differentiation** and **regulate growth**. These compounds are also used for shoot proliferation by the release of axillary buds from apical dominance.

- More commonly used cytokinins are: **benzylamino purine (BAP), isopentenyl-adenine (2-ip), furfurylamino purine (kinetin), thidiazuron (TDZ) and zeatin**

2. AUXINS

- In nature, the hormones of this group are involved with **elongation of stem** and **internodes, tropism, apical dominance, abscission, rooting**, etc.

- In tissue cultures auxins have been used for **cell division, growth and root differentiation**.

- Commonly used auxins are: indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), naphthalene acetic acid (NAA), dichlorophenoxyacetic acid (2,4-D),

3. GIBERRELLINS

- Promote callus growth and elongation of dwarf plants
- Giberrellic acid (GA_3) is the most commonly used gibberellin.

(Ethylene and abscisic acid are other growth hormones that are used.)

IMPORTANCE OF AUXIN TO CYTOKININ RATIO IN PLANT MORPHOGENESIS

High Auxin to Cytokinin ratio: Leads to **Callus initiation, root initiation and embryogenesis**
Low Auxin to Cytokinin ratio: Leads to **Promotes organogenesis and shoot proliferation**

GELLING AGENT

- A gelling agent provides a **solid surface** for the growth of cells . In the absence of gelling agents the tissues will submerge and die due to **lack of oxygen (Anoxia).**

- **Agar** is the most common gelling agent used. It is a polysaccharide obtained from red algae *Gracillaria* and *Gelidium*

- Agar is used at varying concentrations from 0.6 to 0.8%.

- Some other gelling agents used are: Agarose, Gelrite

- Sterile double distilled water is another important component in Plant Growth Medium preparation.

pH of the Culture Medium:

- The pH of the medium should be most ideal for optimal division and Differentiation of cells.
- The optimal pH is considered to be 5.8 ± 0.2.
- In general, a pH higher than 6.0 gives a fairly hard medium and a pH below 5.0 does not allow satisfactory gelling of the agar due to its hydrolysis during autoclaving.
- The pH of the medium is usually adjusted before sterilization.

TYPE OF MEDIUM: SOLID & LIQUID:

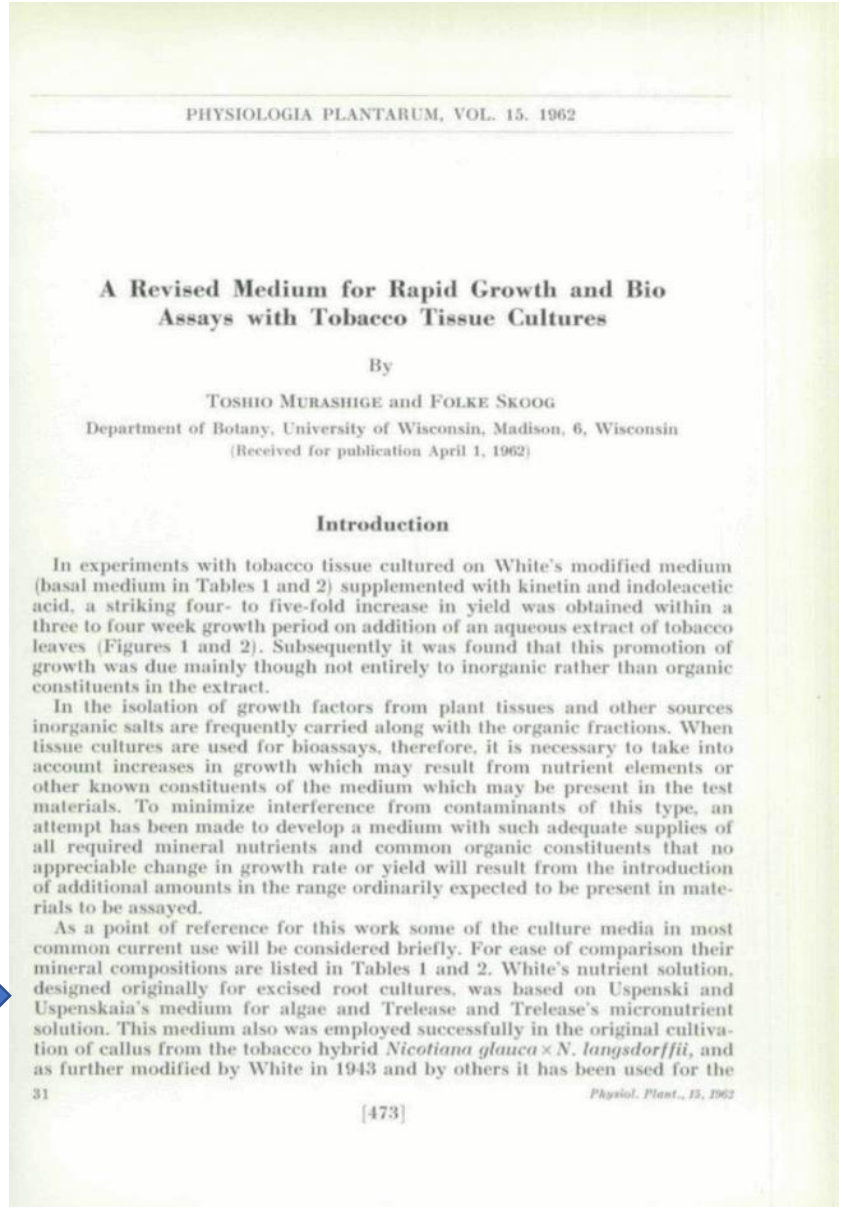
- A medium may be solid or semi-solid or liquid.
- When 0.6 – 0.8% agar is dissolved in the liquid nutrient medium, it makes a solid medium
- Partially solidified medium is known as semi solid when the amount of agar added is reduced
- A medium without agar remains liquid and is known as liquid medium.
- Cultures growing in liquid media are known as suspension cultures

Murashige and Skoog medium 1962 (MS Medium)

TOSHIO MURASHIGE
(Born 1930)

FOLKE SKOOG
(1908-2001)

Classical paper by Murashige and Skoog (1962)



PHYSIOLOGIA PLANTARUM, VOL. 15. 1962

A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures

By

TOSHIO MURASHIGE and FOLKE SKOOG
Department of Botany, University of Wisconsin, Madison, 6, Wisconsin
(Received for publication April 1, 1962)

Introduction

In experiments with tobacco tissue cultured on White's modified medium (basal medium in Tables 1 and 2) supplemented with kinetin and indoleacetic acid, a striking four- to five-fold increase in yield was obtained within a three to four week growth period on addition of an aqueous extract of tobacco leaves (Figures 1 and 2). Subsequently it was found that this promotion of growth was due mainly though not entirely to inorganic rather than organic constituents in the extract.

In the isolation of growth factors from plant tissues and other sources inorganic salts are frequently carried along with the organic fractions. When tissue cultures are used for bioassays, therefore, it is necessary to take into account increases in growth which may result from nutrient elements or other known constituents of the medium which may be present in the test materials. To minimize interference from contaminants of this type, an attempt has been made to develop a medium with such adequate supplies of all required mineral nutrients and common organic constituents that no appreciable change in growth rate or yield will result from the introduction of additional amounts in the range ordinarily expected to be present in materials to be assayed.

As a point of reference for this work some of the culture media in most common current use will be considered briefly. For ease of comparison their mineral compositions are listed in Tables 1 and 2. White's nutrient solution, designed originally for excised root cultures, was based on Uspenski and Uspenskaia's medium for algae and Trelease and Trelease's micronutrient solution. This medium also was employed successfully in the original cultivation of callus from the tobacco hybrid *Nicotiana glauca* × *N. langsdorffii*, and as further modified by White in 1943 and by others it has been used for the

MS MEDIUM: AN INTRODUCTION

MS medium was originally formulated by **Murashige and Skoog in 1962** to optimize tobacco callus bioassay system for facilitating the study of cytokinins. Since then, it is widely used for micro propagation, organ culture, callus culture and suspension culture.

MS BASAL MEDIA: MS medium without growth regulators is called MS Basal medium

Table : Components for Murashige and Skoog's basal medium (MS)

Constituents	Amount (mg L-1)	Amount (mg L-1)
Stock Solution I Macronutrients	Original concentration (1x)	20x Stock Solution
Ammonium nitrate (NH_4NO_3)	1,650	33,000
Potassium nitrate (KNO_3)	1,900	38,000
Calcium chloride $CaCl_2.2H_2O$	440	8,800
Magnesium sulphate $MgSO_4.7H_2O$	370	7,400
Potassium dihydrogen ortho-phosphate (KH_2PO_4)	170	3,400

Constituents	Amount (mg/l)	Amount (mg/l)
Stock Solution II (Micronutrients)	Original Concentration (1X)	200x Stock Solution
Potassium iodide KI	0.83	166
Boric acid H_3BO_3	6.2	1,240
Manganese sulphate $MnSO_4.4H_2O$	22.3	4,460
Zinc sulphate heptahydrate $ZnSO_4.7H_2O$	8.6	1,720
Sodium molybdate $Na_2MoO_4.2H_2O$	0.25	50
Copper sulphate pentahydrate CuSO4.5H2O	0.025	5
Cobalt chloride hexahydrate $CoCl_2.6H_2O$	0.025	5
Stock Solution III (Iron)	Original concentration	200x Stock Solution
Ferrous sulphate heptahydrate $FeSO_4.7H_2O$	27.8	5,560
Di Sodium EDTA dihydrate $Na_2EDTA.2H_2O$	37.3	7,460

Constituents	Amount (mg L-1)	Amount (mg L-1)
Stock Solution IV (Organic Nutrients; 200x)	Original concentration	200X Solution
Inositol	100	20,000
Nicotinic acid	0.5	100
Pyridoxine. HCl	0.5	100
Thiamine. HCl	0.1	20
Glycine	2	400
Carbon source		
Sucrose	3%	

PREPARATION OF MS MEDIA

- Easiest method: Dissolve company made ready to use medium mix into distilled water. It can be supplemented with sucrose, agar, auxins (IAA) and cytokinins (Kinetin) to generate a complete medium for growth of plant tissue culture.

Steps in the Preparation of Culture Medium

1. Prepare stock solutions: For MS basal medium, four stock solutions are prepared. For individual stock solution weigh the required quantity of nutrient, dissolve in double distilled water (ddH_2O). Each component should be separately dissolved to the last particle and then mixed with the others. Make up the volume to 1 liter. Refrigerate and store the stock solution in refrigerator.

Stock Solution I Macronutrients (20X)
Ammonium nitrate (NH_4NO_3)
Potassium nitrate (KNO_3)
Calcium chloride dihydrate $CaCl_2.2H2O$
Magnesium sulphate heptahydrate $MgSO_4.7H2O$
Potassium dihydrogen ortho-phosphate (KH_2PO_4)

Stock Solution II (Micronutrients)(200X)
Potassium iodide KI
Boric acid H_3BO_3
Manganese sulphate tetrahydrate $MnSO_4.4H_2O$
Zinc sulphate heptahydrate $ZnSO_4.7H_2O$
Sodium molybdate dihydrate $Na_2MoO_4.2H_2O$
Cupric sulphate pentahydrate $CuSO_4.5H_2O$
Cobalt chloride hexahydrate $CoCl_2.6H_2O$

Stock Solution III (Iron)(200X)
Ferrous sulphate heptahydrate $FeSO_4.7H2O$
Di Sodium EDTA dihydrate $Na_2EDTA.2H2O$

Stock Solution IV (Organic Nutrients; 200x)
Inositol
Nicotinic acid
Pyridoxine. HCl
Thiamine. HCl
Glycine

Protocol to prepare 1 liter of medium

(1) Dissolve 30 gms sucrose in 200 ml ddH_2O (double distilled water).

(1) Take ddH_2O in another flask and add in sequence appropriate amount of stock solutions.

C_1x V_1= C_2x V_2 where C_1 is the stock concentration, V_1 is the Volume (ml) of stock required to be added in the media, C_2 is the final concentration of the stock and V_2 is the final volume of the media.

20x X ? = 1x X lLiter 0.05 l= V1

S.No	Stock Name	Stock Concentration	Stock solution volume required for 1 liter medium
1.	Stock Solution I	20X	0.05 L = 50ml
2.	Stock Solution II	200X	0.005L= 5ml
3.	Stock Solution III	200X	5ml
4.	Stock Solution IV	200X	5ml

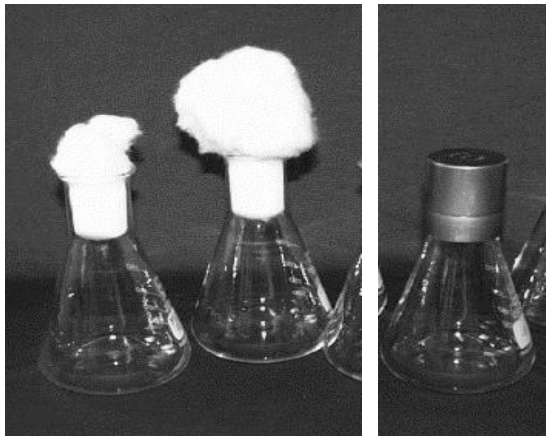
3. Pour the sucrose solution and the mix of stock solutions into 1 litre measuring cylinder. Make up the volume to 1 litre by adding double distilled water. Shake well to mix up uniformaly.

4. Adjust the pH of theh liquid medium 5.8±0.2 with the aid of 0.1N HCl or 0.1N NaOH. This is done by using pH meter.

5. Add 0.6% - 0.8% agar to the liquid medium to make it solid. Heat to 60 degree Celsius to dissolve the agar particles completely. If liquid media is required, do not add agar.

6. Dispense the culture media into culture tube(20 ml/tube) or wide mouth conical flask (25-40ml/ flask).

7. Insert a non absorbant cotton plug wrapped in gauge cloth. Cover the plug with the help of paper and rubber band .



8. Medium is finally sterilized by autoclaving under steam at a pressure 15lb/in^2 and a temperature of 120^{0}C for 15 minutes. This process is carried out in autoclave or even regular pressure cooker.

Aseptic techniques in *in vitro* culture – sterilization – different methods

– sterilization of instruments and glassware, medium, explants; working principle of laminar air flow and autoclave

Aseptic techniques in *in vitro* culture- sterilization

INTRODUCTION

- Culture medium is rich in sugar and other nutrient, hence is optimal for rapid growth of microorganisms like fungi and bacterial
- In initial stages the microbes may release toxic waste which will retard the growth of cultured tissue
- Later microorganisms outgrow the cultured tissues very rapidly and kill the plant tissues.

Sources of contamination

- labware or culture medium,
- plant material or
- environment in the laboratory,
- instruments used for culture or
- the operator/human

Hence successful plant tissue culture demands maintainence of an almost aseptic environment which includes proper sterilization of plant materials, nutrient media, culture rooms, instruments, culture vessels,instruments etc to make them microbe free.

Killing or removing all forms of microbial life (including endospores) in a material or an object is defined as the process of sterilization.

Different Methods of sterilization

1. Dry heat technique:

- Dry heat technique uses hot air for sterilization

- Sterilize Glassware (like petri dishes, flasks, pipettes, and test tubes), plastic wares(of specific composition), instruments like needle, forceps, scalpel, etc

- Two instruments that use Dry heat technique are
  i) Hot Air Oven: Glasswares can be sterilized at 160°C to 180°C for nearly three hours.
  ii) Glass bead sterilizer: use dry heat upto 300°C . Are used for sterilizing laboratory instruments like forceps, scalpel etc.

i)

ii)

2. **Flame sterilization**:

The oldest method around is flaming. Flame sterilization means holding an instrument in an open flame after dipping it in 95% alcohol.
Is a common method done for sterilizing inoculation loop, forceps, scalpels etc.
The mouth of the culture vessels are also sterilized by this method prior to inoculation or subculture

3. **Surface sterilization:**

- Plant surfaces harbour a wide range of microbial contaminants. This source of infection can be avoided by surface sterilization of the plant material before planting it on the nutrient medium.

- Plant tissues can be surface sterilized using various sterilants

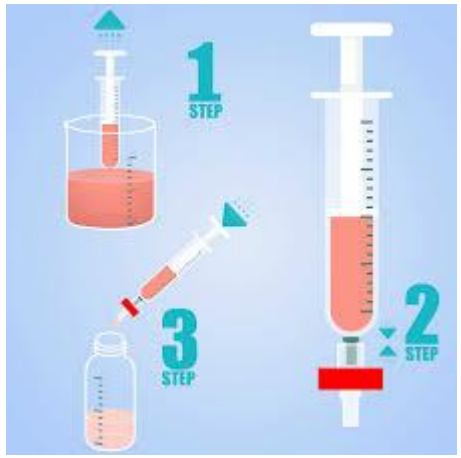
Sterilizing agent	Concentration (%)	Duration (min)	Effectiveness
Calcium hypochlorite	9–10	5–30	Very good
Sodium hypochlorite	0%(v/v)	5–30	Very good
Hydrogen peroxide	10–12	5–15	Good
Bromine water	1–2	2–10	Very good
Silver nitrate	1	5–30	Good
Mercuric chloride	0.1–1	2–10	Satisfactory
Antibiotics	4–50 mg L-1	30–60	Fairly good

4. Wiping with 70% alcohol

Wiping with 70% alcohol is carried out to sterilize platforms of laminar air flow cabinet, the walls , ceilings, doors and windows of tissue culture related rooms and hands of the tissue culturist or operator which cannot be sterilized by other methods

5. Filter sterilization

- Some of the plant growth regulators (e.g. GA3, zeatin, ABA) urea, certain vitamins, pantothenic acid, antibiotics, colchicine, plant extracts and enzymes used in tissue culture are thermolabile.
- The solution of the thermolabile compound is sterilized by membrane filtration and added to the autoclaved medium when the latter has cooled to around 40^0C.
- For filter-sterilization of a solution, bacteria-proof membranes of pore size 0.22–0.45 µm are used.



7. Autoclaving: Mainly involves heating material to 121^0C at 15 p.s.i (pound per square inch) for different intervals of time depending on the volume of the medium used for autoclaving.

8. Laminar Air Flow Chamber: Is used for creating aseptic working space by blowing filter sterilized air through enclosed spaces.

AUTOCLAVE

- Autoclave is an instrument used for sterilizing other equipments and tissue culture medium by heating with pressure saturated steam. (Gk. *Autos* : self; *clavis* : key)
- 1897: Charles Chamberland developed the autoclave as a sterilization alternative to open flame technique.
- Autoclaves are also called steam sterilizers.

Working Principal:

Temperature of a gas increases corresponding to an increase in pressure and also the penetration power of the water molecules in steam increases when they become more energized.

Temperature is directly proportional to pressure

•Water usually boils at 100°C under normal atmospheric pressure ; however, the boiling point of water increases if the pressure is increased.

•The high pressure increases the boiling point of water and thus helps achieve a higher temperature for sterilization.

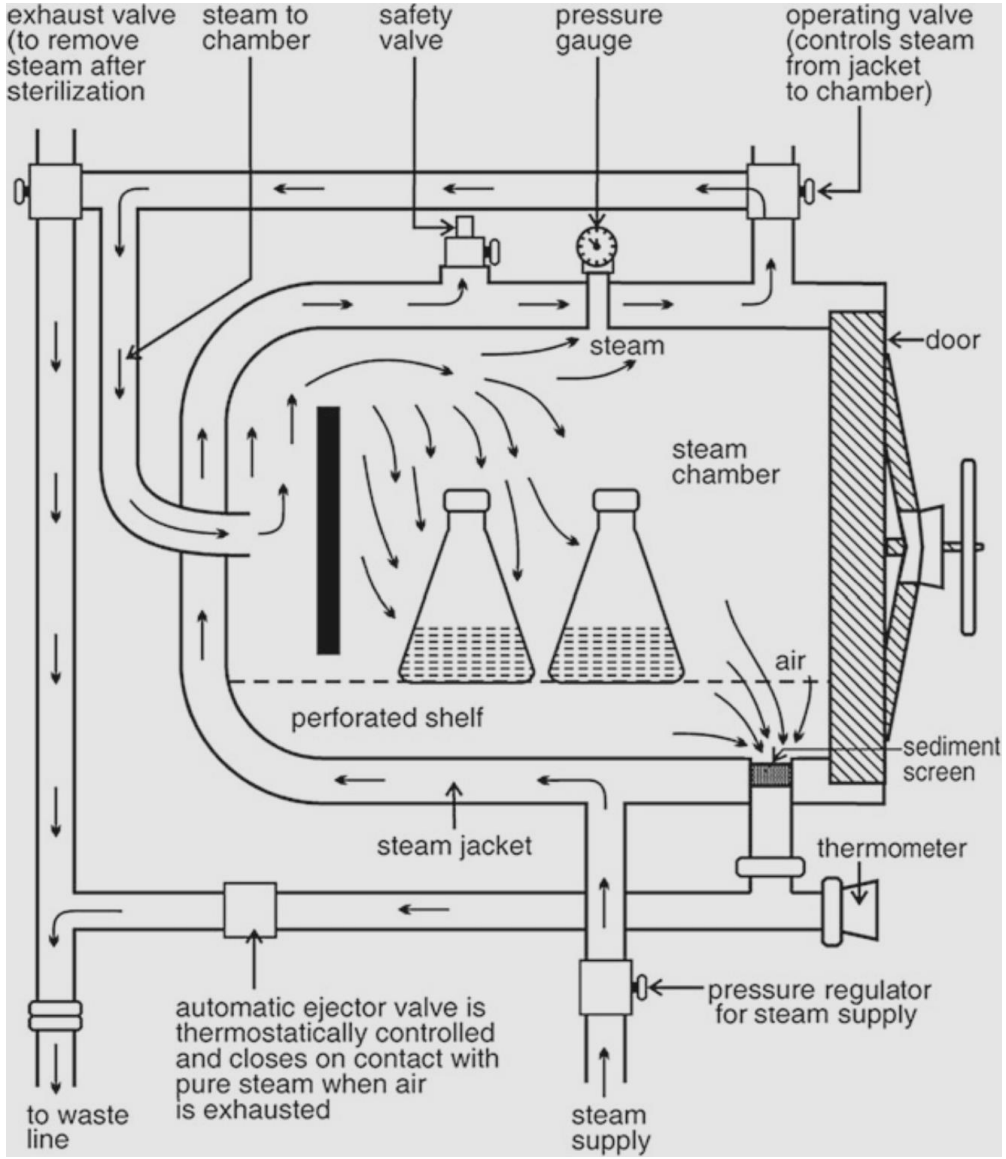
- This principle is employed in an autoclave where the water boils at 121°C at the pressure of 15 psi .

•Similarly, the high pressure also facilitates the rapid penetration of heat into deeper parts of the material, and moisture present in the steam causes the coagulation of proteins causing an irreversible loss of function and activity of microbes.

•When this super heated steam comes in contact on the surface, it kills the microbes.

- Once the sterilization phase is completed (which depends on the level of contamination of material inside), the pressure is released from the inside of the chamber through the whistle.

- The pressure inside the chamber is then restored back the ambient pressure while the components inside remain hot for some time.


exhaust valve (to remove steam after sterilization
steam to chamber
safety valve
pressure gauge
operating valve (controls steam from jacket to chamber)
steam
door
steam chamber
air
perforated shelf
sediment screen
steam jacket
thermometer
automatic ejector valve is thermostatically controlled and closes on contact with pure steam when air is exhausted
pressure regulator for steam supply
to waste line
steam supply

LAMINAR AIR FLOW: WORKING PRINCIPLE

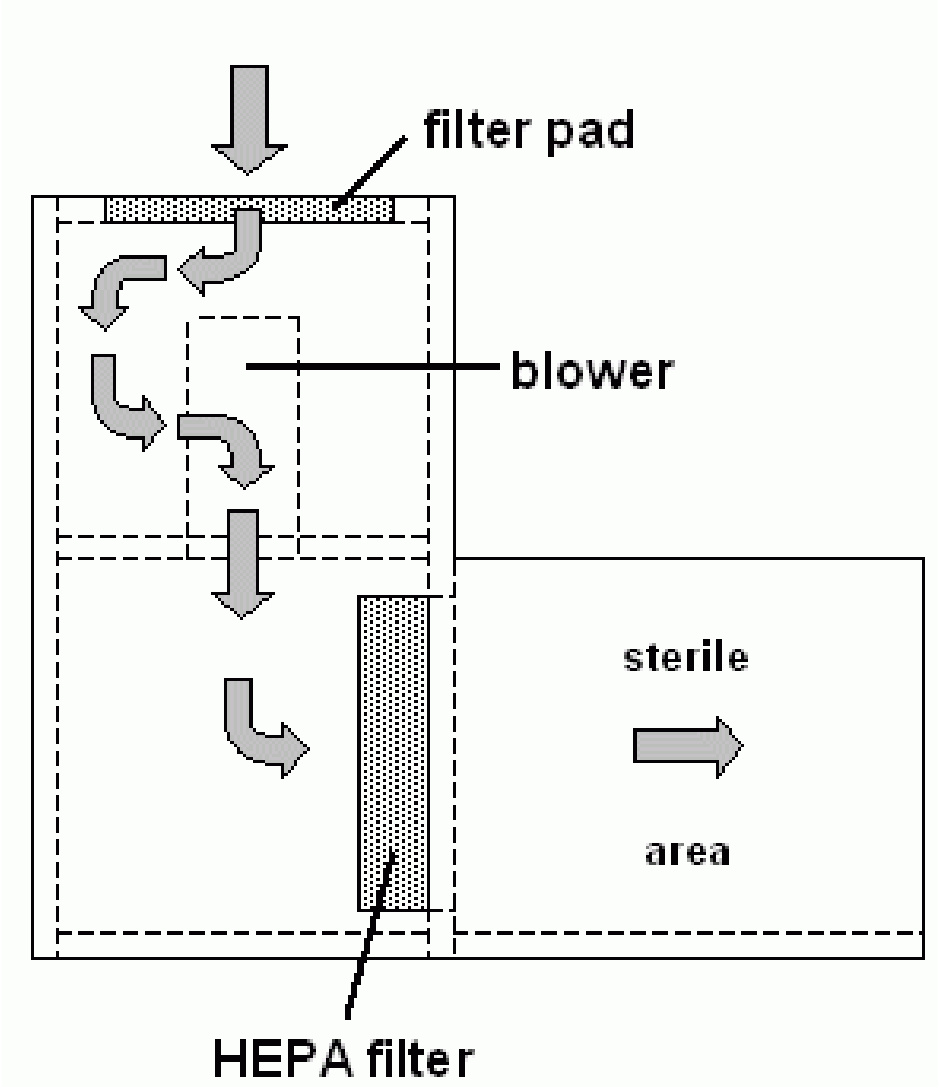
- Sterilization technique employed for creating an aseptic working environment by blowing filter sterilized air through an enclosed space which is open on one side. This is carried out with the help of laminar air cabinet

- Air is first filtered by passing it through a membrane filter to remove large particles .

- The filtered air is then passed through a high- efficiency particulate air filter (HEPA FILTER) which filters out all particles larger than 0.3µ.

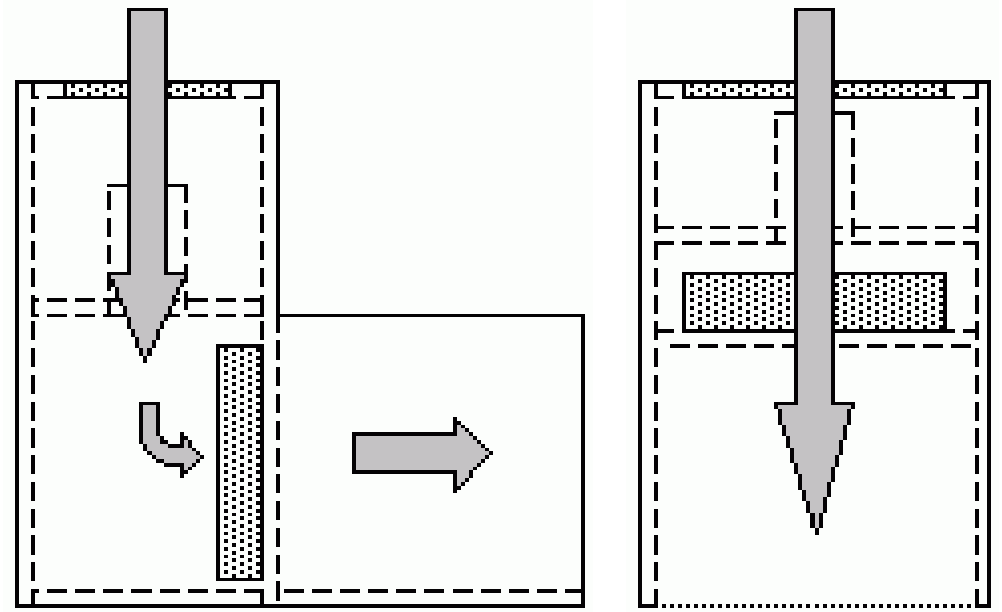
- This filter sterilized air is blown through the cabinet at a speed of 1.8 km per hour with the help of blower motors. This makes the enclosed working area aseptic.

Side view of a laminar flow hood



The two types of laminar flow hoods: based on the direction of the air flow

1. **Horizontal:**When you use a flow hood with horizontal air flow the air moves from the back of the working area to the front.
2. **Vertical :**In a vertical flow the air moves from the top of the working area to the bottom and leaves the area through holes in the base.



a) Horizontal

b) Vertical

different methods – **sterilization of instruments and glassware**

Most commonly use two methods for sterilization of instruments and glassware like are

1. Dry heat sterilization

2. Autoclaving

Sterilization of Medium:

1. Autoclaving
2. Filter Sterilization

Sterilization of Explants:

1. By using surface sterilization procedures using chemical sterilants.

4. Preparation of explants– surface sterilization, inoculation, incubation, subculturing.

What are Explants?

The plant part to be cultured *in vitro* on a nutrient medium is known as Explant.

I) Explant Selection:

The following factors are crucial in explant selection:

1. Age of the organ that is to serve as the explant source
2. Season in which the explant is obtained
3. Size and location of the explant
4. Quality of the source plant
5. Ultimate goal of cell culture
6. Plant genotype

1. EXPLANT AGE: Is a very important factor to be considered

- physiologically younger tissue is generally more responsive *in vitro*.
- In many cases, older tissue will not form callus that is capable of regeneration
- younger tissue is usually the newest formed and is generally easier to surface disinfect and establish clean cultures.

2. SEASON

- The season of the year can have effects on contamination and response in culture.
- For example, buds or shoots taken during the spring of the year (February to march) when the shoots are in a flush state of growth are more responsive than dormant buds.
- As the season of the year passes from spring, summer, and fall to winter the explant generally does not respond as well in culture.
- Additionally, contamination rates also increase as the summer progresses; rainy season and winter contamination can increase to greater percentage

3.EXPLANT SIZE

The explant size has an effect on the response of the tissue (usually optimal 2 -5 mm).

- ❑ Generally, the smaller the explant, the harder it is to culture.
- ❑ The culture medium should also have additional components.
- ❑ The larger explants probably contain more nutrient reserves and plant growth regulators to sustain the culture.
- ❑ Plants have different hormonal balances throughout the plant and depending on the location of the explant, the explant can have a different endogenous level of plant growth regulators. Internal differences in hormone balance in the tissue can result in varying *in vitro* responses.

4. PLANT QUALITY

- ❑ Obtain explants from plants which are healthy as compared to plants under nutritional or water stress or plants which are exhibiting disease symptoms

5. GOAL

- Depending on what type of a response is desired from the cell culture, the choice of explant tissue will vary. Any piece of plant tissue can be used as an explant (Fig 1)

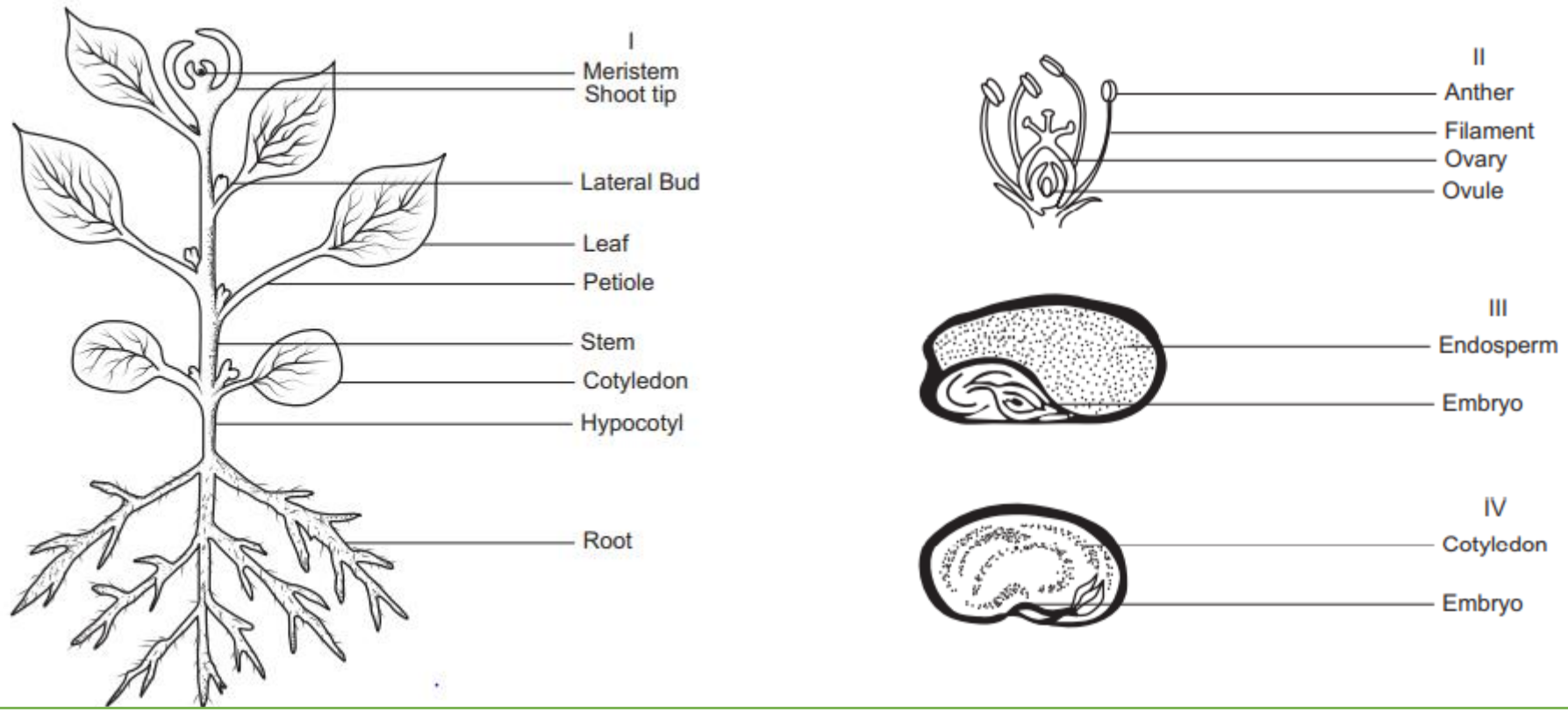


Fig 1:Schematic drawings of (I) a plant, (II) a flower, and (III) monocotyledenous and (IV) dicotyldenous seeds indicate potential explant tissues.

For example, if the goal is

GOAL	EXPLANT USED
clonal propagation	lateral or terminal bud or shoot
callus induction	pieces of the cotyledon, hypocotyl, stem, leaf, or embryo, seedling tissues from aseptically germinated seeds or immature inflorescences
protoplast isolation	Leaf tissue from the aseptically germinated seed
haploid plants or callus	the anther or pollen

6. **GENOTYPE**

- Within plant genus, different species or cultivars exhibit differences in the genotypes (genotype refers to genetic constitution of an organism)
  (eg. Coffea: genus, *Coffea arabica*, *Coffea canephora* var Robusta cv. S274)

- Some genotypes are not responsive in culture, or recalcitrant, while others easily respond to produce callus or shoots.

- Testing numerous genotypes of a crop or ornamental species is generally a major experimental parameter to identify those that will respond in culture.

- *In vitro* **recalcitrance** is the inability of plant cells, **tissues** and organs to respond to **tissue culture** manipulations.

II EXPLANT STERILIZATION

- The sterilizing of plant materials is achieved in various ways.

a) Explants like meristems, leaf tissues, bulbs, or any other part from plants produced under non-aseptic conditions are sterilized .

b)The most convenient procedure is to produce the explant materials under sterile conditions, such as from aseptically germinated seedlings. (Seed Sterilization) The tissues to be used are then removed and are immediately ready for use.

a) STERILIZATION OF PLANT MATERIAL

- Plant materials to be cultured, should be surface sterilized to remove surface borne microorganisms.
- The procedure is carried out in sterile condition, either in front of a Laminar air flow or inside inoculation chamber.

Typical sterilization protocol involves the following steps:

Steps to be carried out in general laboratory:

1. Thoroughly wash the explant in tap water and immerse in 5% v/v solution of liquid detergent for 10- 15 minutes. Again wash thoroughly in tap water, followed by rinse in distilled water. This procedure is very beneficial for stem, leaf, and shoot tip explants from the field or greenhouse because it removes surface contaminants.

Steps to be carried out in laminar air flow or sterilized inoculation chamber

1. The explants are dipped in 70% ethyl alcohol for 60 secs.

2. Immediately transfer the explant into autoclaved bottle and pour 0.1% mercuric chloride or 5-10% sodium hypochlorite(v/v) solution (freshly prepared). Keep for 5-15 mins. The bottle is frequently swirled so that surfaces of explant come equally in contact with sterilant.

3. Decant the sterilant and wash the explant thoroughly with several changes of autoclaved distilled water to remove all traces of sterilants.

4. The explants are ready for inoculation.

Problem Solving :

- If tissues turn brown or white, they are oversterilized and the cells are dead. Reduce the length of time in ethanol or bleach, or reduce the concentration of sterilizing agent.

- If tissues are highly contaminated, the sterilization treatment needs to be more stringent or alternate treatments need to be tested. Mercuric chloride can be more effective than the hypochlorite treatments. The chlorine gas is very penetrating, but can also reduce the viability of seeds.

- Testing is necessary to determine the balance between obtaining sterility and retaining the viability of the seeds or tissues.

Detection of Contamination:

A general and effective method would be to use the potato dextrose powder. The media can be prepared as a liquid or an agar and is effective in detection of bacteria, yeast, and fungi.

1. Prepare the potato dextrose medium as agar plates or as a liquid in test tubes.
2. Transfer the materials to be tested to the potato dextrose media.
3. Incubate for 24 h at 37°C

If the sterilization procedure is not efficient, growth of bacteria, yeast or fungi will be visible on the media petriplates.

III) EXPLANT INOCULATION

Inoculation means transferring of the surface sterilized plant material onto the nutrient media.

To prevent entry of any micro-organisms at the time of inoculation, all procedures should be carried out under aseptic condition.
Procedure for inoculation:

1. Keep all the sterilized articles (media, instruments, glass containers etc) for inoculation in the inoculation chamber or laminar air flow.

2. Switch on the UV lamps of inoculation chamber/laminar air flow for one hour.

3. In the case of laminar air flow, after switching off the UV lamp, switch on the air flow to blow air for 15 minutes before work.

4. The working top surface of the inoculation chamber should be wiped with alcohol before starting the work.

5. Wear a clean apron and use a mask. Clean the hands with alcohol and dry it.

6. Pour 70% alcohol in a sterile jar and dip all the instruments into it. Light the spirit lamp.

7. Take the surface sterilized or aseptic plant material in a sterile petri dish.

8. Remove the cotton plugs of the culture tube or flask and flame sterilize the neck of the culture tube or flask.

9. Transfer the tissue onto the medium and replace the cotton plug.

10. After each usage the instruments like forceps are passed through the flame of the spirit lamp.

PRECAUTIONS:

1. Always air dry the hands after wiping with alcohol and keep the alcohol moistened hands away from spirit lamp.

2. Always enter the inoculation chamber only 15-30 minutes after switching off the UV lamp. Exposure to UV light builds up a high concentration of ozone gas inside the chamber which is toxic.

3. Do not use instruments that are hot after flame sterilization to hold or cut the plant material.

4. Don’t heat the neck of the glass containers excessively.

5. Also do not keep the media exposed for a long time for inoculation of explants.

IV) EXPLANT INCUBATION

Optimal Culture room conditions are essential for proper growth of the plants in tissue culture:

- Temperature, Humidity, Light duration and intensity are important factors

1. After inoculation the tissue onto culture medium, the cultures are incubated on culture racks at constant ambient temperatures of 25-28^{0}C.

2. Humidity: Is maintained at 40% in most cases.

3. Light duration: Depending on culture condition. Eg: Coffee Seed germination: complete dark condition is maintained. For most plants 16hrs of light and 8 hrs of drak photoperiod is generally used.

4. Light intensity: illumination is provided by cool white fluorescent light placed approximately 18 inches above culture to give light intensity of 4-10 x 10^3 lux.

III) EXPLANT SUBCULTURING

Neccessity for subculturing:

1. The nutrient medium is exhausted (deficiency phenomena).
2. The nutrient medium dries out (resulting in too high salt and sugar concentrations).
3. Growth has filled the tube or flask.
4. The material is needed for further propagation
5. Brown and/or black colouring appears in the agar: plant tissues sometimes give off toxic substances during the first few weeks, which diffuses into the agar or liquid medium.
6. It is needed to give the isolated material a different growth and development pattern, on a known nutrient medium.
7. The medium has become liquid due to a lowering of the pH by the plant.

Sub-culturing is carried out as follows:

1. The tube or flask is externally sterilized with 95 % alcohol (on a cotton wad).

2. Any aluminium foil or film and then the cotton plug are removed from the tube or flask in the laminar air-flow cabinet.

3. The explant or callus clump is taken out and put in a sterile Petri dish or on (between) sterile filter paper.

4. After cutting out the material is inoculated onto a new nutrient medium. When cutting pieces out, strong healthy homogeneous (not necrotic etc.) material is selected.

5. Micropropagation - Different methods – apical, axillary bud proliferation, direct and indirect organogenesis and somatic embryogenesis.

Different phases of micropropagation

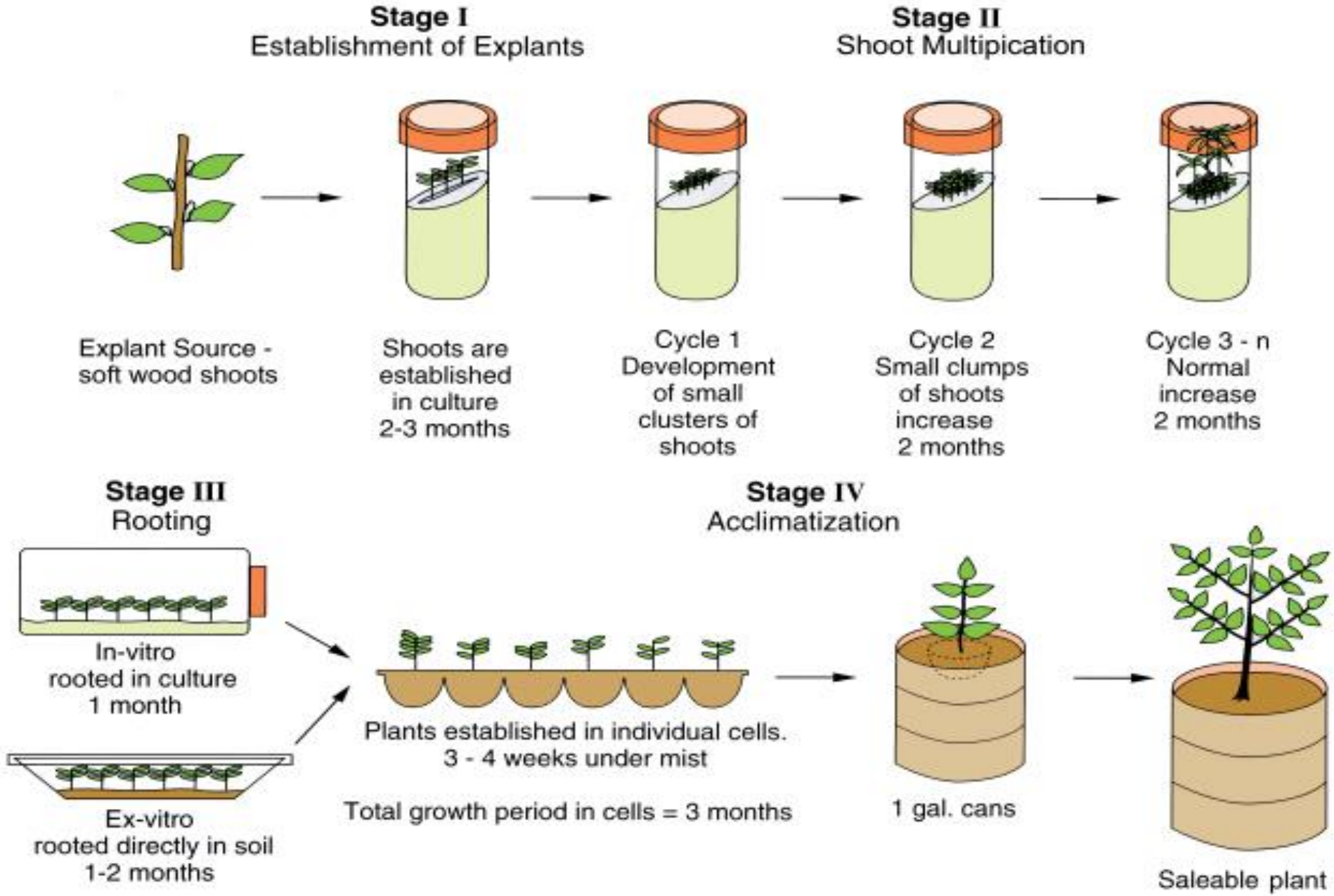
- **Clonal Propagation**: The production of genetically identical plants by vegetative or asexual method is called CLONAL PROPAGATION

- **Micropropagation**: Clonal propagation *in vitro* is called Micropropagation.

- Stages of Micropropagation: In vitro clonal propagation is a complex process and is completed in five stages:

  i. Stage 0
  ii. Stage I
  iii. Stage II
  iv. Stage III
  v. Stage IV

  } *in vitro* conditions



source: rrecenvhort.ifas.ufl.edu

i) Stage 0: Mother plant selection and preparation

- The first step in micropropagation is the selection of stock or mother plants for their multiplication on a large scale.
- A disease free mother plant with desirable qualities are selected.
- The selected stock/mother plants they are maintained in controlled environmental conditions of low humidity, irrigation, and without any systemic microbial infection for a period of 3 months for culture initiation.

ii) Stage I: Aseptic Culture Establishment

Initial stage of micropropagation and it involves initiation and establishment of aseptic culture cultures. It is completed in four stages:

a) Explant isolation
b) Explant preparation
c) Inoculation
d) Establishment of cultures

a) Explant Isolation: Separation of explants from the selected stock plant.

a) Explant Preparation: Surface sterilization of the isolated explant with ethyl alcohol, mercuric chloride or sodium hypochlorite.

b) Inoculation: Introduction of sterilized explant to suitable culture medium

a) Establishment of culture: Post inoculation proper healthy growth of explants in optimal media and physical environmental condition

- Stage I lasts for usually 3 months to 2 years.
- Requires at least four passages of subculture.

Two major problems associated with stage I

a) Microbial contamination: Proper surface sterilization methods should be implemented

b) Phenolic Exudation: the effect can be minimized by adding activated charcoal to the medium or by adding certain antioxidant chemicals to the medium like citric acid.

iii) Stage II: Multiplication of the Explants

- Multiplication of explants takes a considerable time through regeneration of shoots from explants.
- It lasts 10- 36 months
- Cytokinin-rich media are used to inoculate nodal explants for multiple shoot proliferation

Stage II is often accomplished by four different ways:

a) Callus mediated shoot multiplication
b) Apical or axillary shoot proliferation
c) Adventitious shoot proliferation
d) Direct somatic embryogenesis

iv) Stage III : In vitro rooting

- Involves transfer of shoots to rooting medium
- The shoots or plantlets proliferated in stage II are small and devoid of roots
- In vitro rooting is induced by addition of auxins to the culture medium
- Cytokinin generally inhibits rooting and hence the cytokinin concentration should be kept low

v) Stage IV: Transplantation or Hardening

- In the hardening stage the plantlets of stage III are prepared for external soil conditions from their in vitro conditions. The plants are acclimatized in green house condition in suitable soil mix in pots under controlled conditions of temperature, light and humidity
- This involves the plants becoming resistant to stress, moisture, and disease resulting in the autotrophic nature of plants from the heterotrophic nature in in vitro culture conditions.
- Protection must be given to plantlets from direct sunlight and a decrease in relative humidity should be done over a period of time.
- Well-developed roots are formed by the plantlets during this acclimatization period and cuticular wax is also formed in the aerial tissues.
- After this the plantlets become suitable for transfer to the open fields

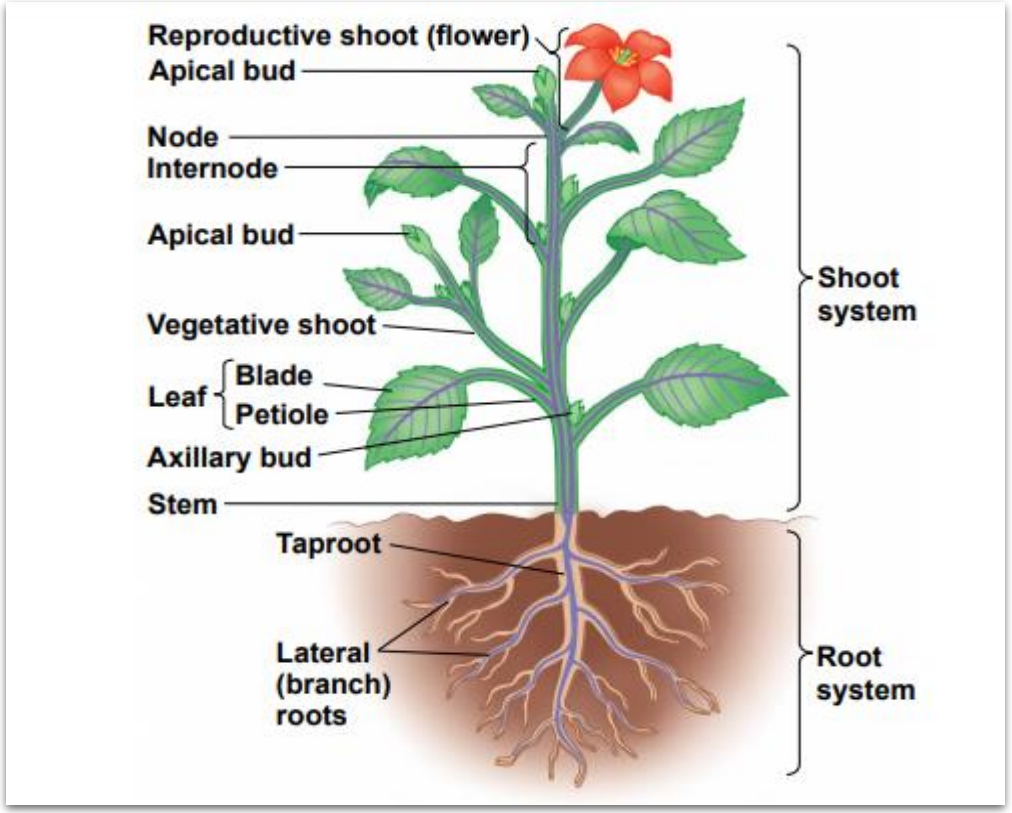
Fig: Banana micropropagation
Shoot tip culture for banana micropropagation: a. sword sucker and explant; b. shooting after apical disabling; c. proliferation; d. multiple shooting; e. rooting; f. nursery hardening (Source: Singh et al 2011)

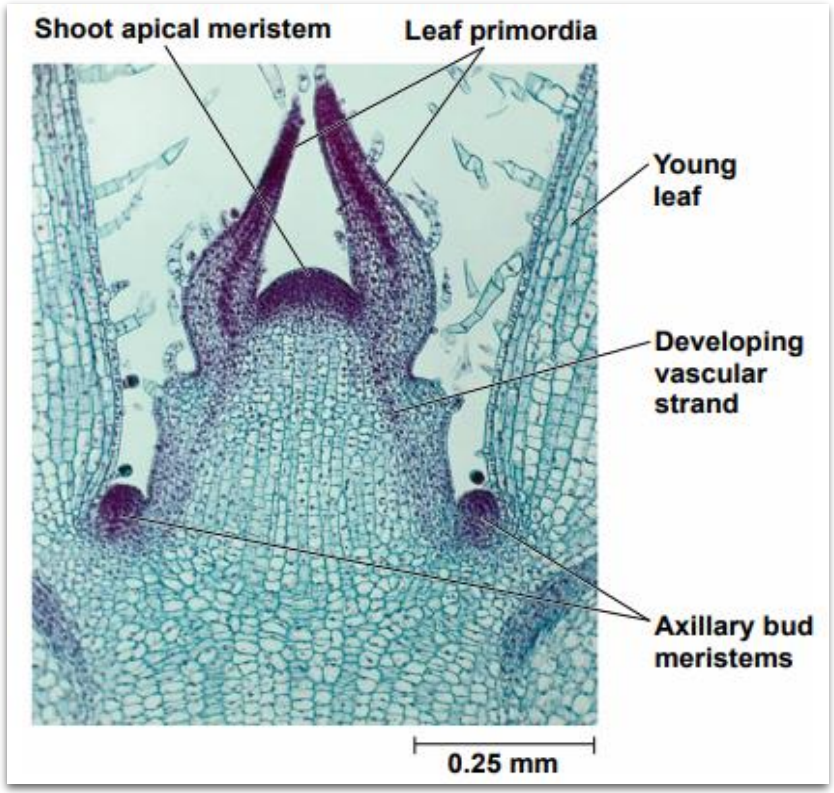
METHODS OF MICROPRAPAGATION

1. Axillary bud proliferation:

2. Organogenesis

3. Somatic embryogenesis





Axillary bud: Bud located in the axil of a leaf. It is an embryonic shoot that lies at the junction of the stem and petiole that gives rise to a branch or flower

Apical Bud : Bud located at the terminal or apical location of stem or branch

Node : Points of attachment for leaves, branches, and flowers

Internode: A section of stem between two stem nodes

Meristem: A region of plant tissue, found chiefly at the growing tips of roots and shoots and in the cambium, consisting of actively dividing cells forming new tissue

1. Multiplication by axillary bud proliferation:

- Micropropagation by the proliferation of axillary buds involves the stimulation of axillary buds to develop to shoots.
- The production of plants from axillary buds or shoots has proved to be the most generally applicable and reliable method of true-to-type *in vitro* propagation.

- Micropropagation by axillary bud proliferation is accomplished in three principal ways:

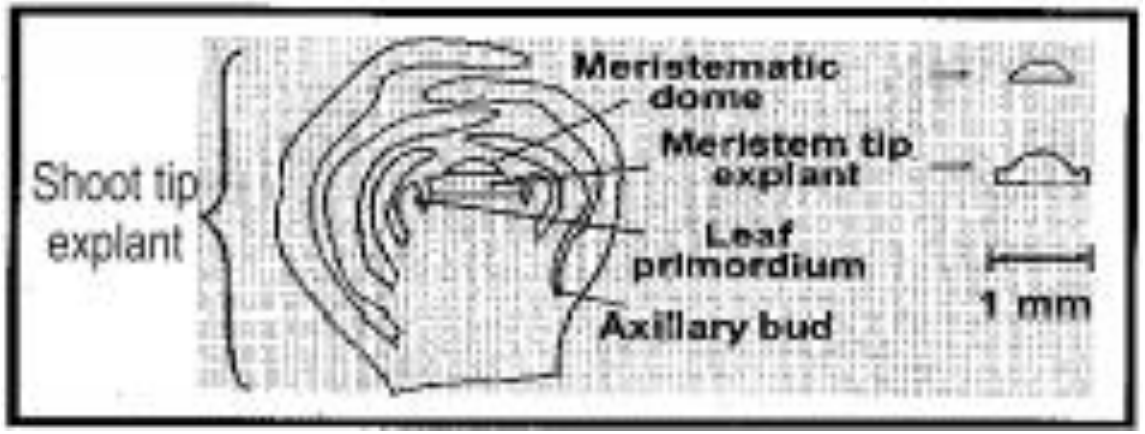
i. Meristem Culture
ii. Shoot tip culture
iii. Single node culture

Shoot Tip Culture:

Shoot tip culture is described as the culture of terminal (0.1- 1.0 mm) portion of shoot comprising the meristem (0.05 -0.1mm) together with primordial and developing leaves and adjacent stem tissue.

Meristem culture:

Meristem culture is the in vitro culture of a generally shiny special dome-like structure measuring less than 0.1mm in length and only one or two pairs of the youngest leaf primordia, most often excised from shoot apex.



i) Meristem culture:

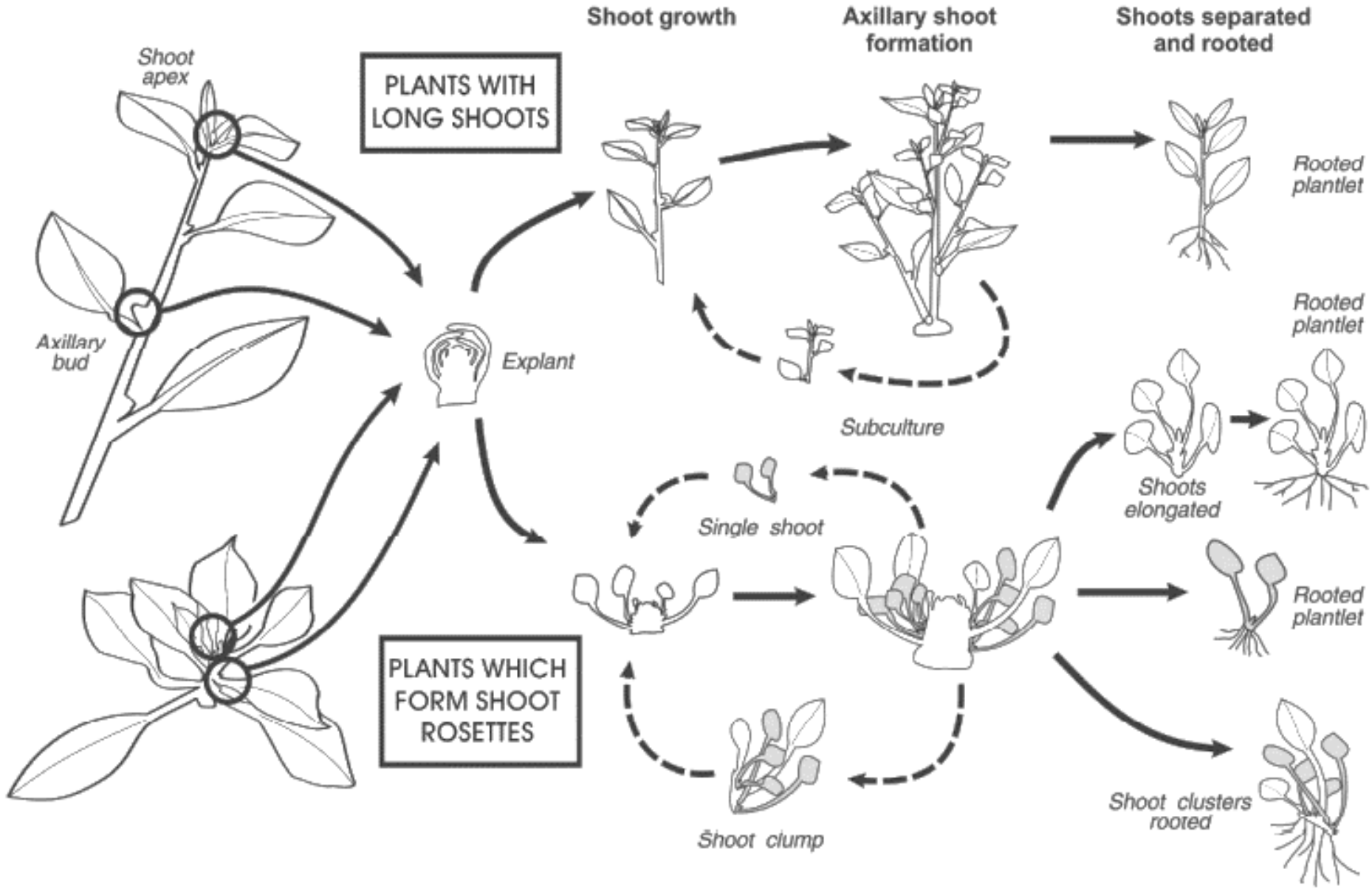
- Micropropagation using meristems is called meristem culture, meristem tip culture or mericulture.
- Meristem culture *in vitro* is used for the elimination of viruses and related pathogens
- Apical meristems are dome of totipotent cells which are capable of active division and differentiation to form specialized permanent tissues of shoots and roots.
- For meristem culture, very small explants (0.05 to 0.1 mm) are taken from terminal buds, with the help of scalpels and stereomicroscopes.
- Each explant must consist of the meristematic dome and one or two leaf primordium
- The meristematic dome is the center of proliferation, growth and differentiation of cells and development of tissues and organs.

ii) Shoot tip culture

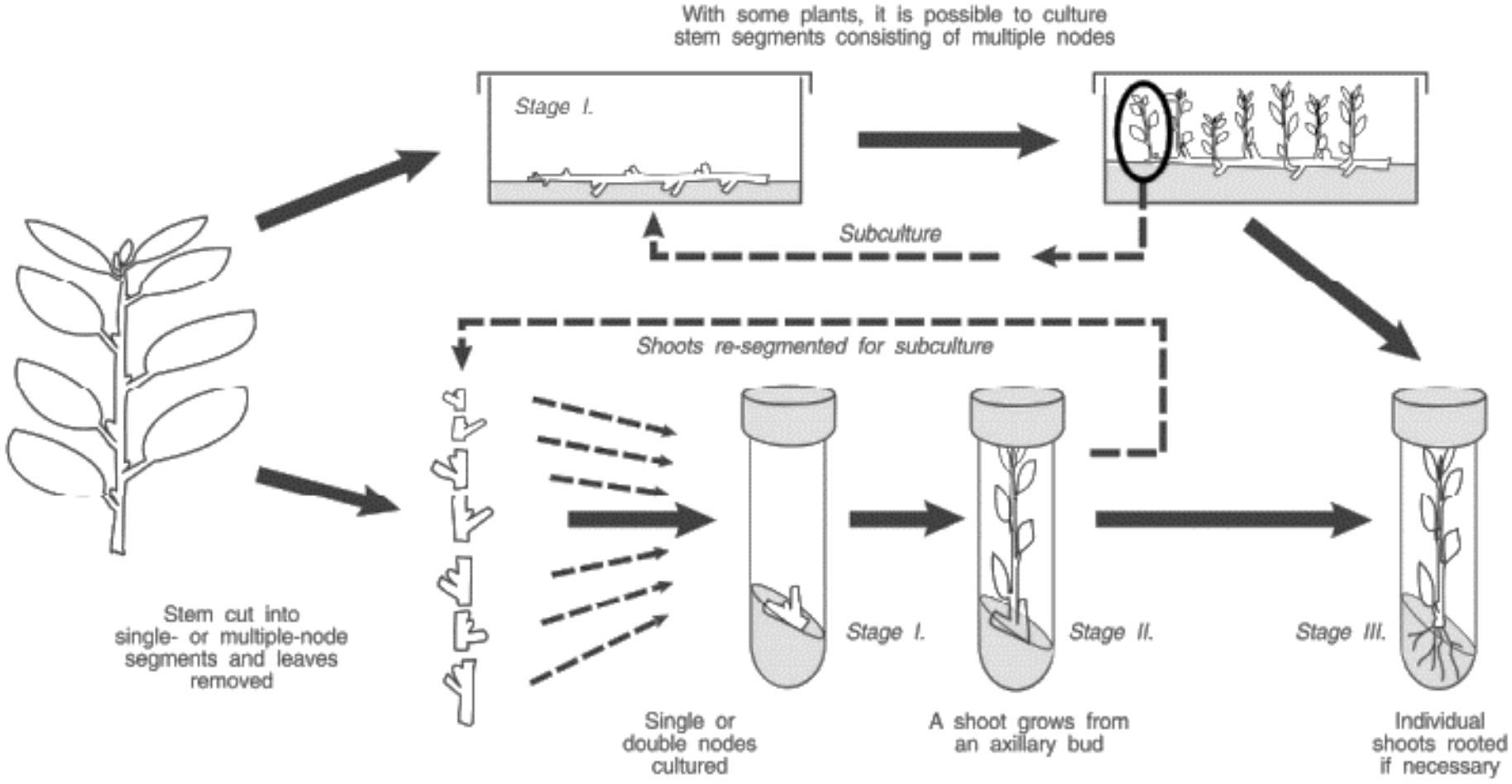
- The apical meristem together with one to three pairs leaf primordia constitute the shoot apex.

- For shoot tip culture relatively large explants consisting of the shoot apex and small unexpanded leaves at different stages of development are sliced away from the stem by applying V shaped cuts with the help of sterilized knife.

- The dominance of apical meristem is arrested and the development of axillary shoots is stimulated by the addition of appropriate growth regulators like cytokinin to the culture medium.


Shoot growth
Axillary shoot formation
Shoots separated and rooted
PLANTS WITH LONG SHOOTS
Shoot apex
Axillary bud
Explant
Rooted plantlet
Rooted plantlet
Subculture
Shoots elongated
Single shoot
Rooted plantlet
PLANTS WHICH FORM SHOOT ROSETTES
Shoot clusters rooted
Shoot clump

iii) Node culture:



Organogenesis:

- The induction of different adventitious organs of the plant from cultured tissues under *in vitro* condition is known as organogenesis.
- The organogenesis includes two steps, caulogenesis and rhizogenesis.
- Induction of adventitious shoot buds from the cultured tissues is known as caulogenesis.
- Induction of adventitious roots from the cultured tissue is known as rhizogenesiss
- Rhizogenesis is induced only after caulogenesis in plant tissue culture methods.

Plant Production through organogenesis can be achieved by two modes-

(i) Direct Organogenesis:

Emergence of adventitious organs directly from the explant. The adventitious buds may arise directly from the tissue of the explant and not from callus

(ii) Indirect Organogenesis:

Organogenesis through callus formation. The steps involved in Indirect organogenesis are:

a) Dedifferentiation of the explant
b) Initiation and Proliferation of callus tissues along the cut edges of the explant
c) Induction of new organs from the newly formed callus (redifferentiation)

Exogenously supplied phytohormones control and promote organogenesis:

Auxin /Cytokinin- 10:1-100:1 induces root
Auxin/Cytokinin- 1:10-1:100 induces shoot

Intermediate ratios of around 1:1 Auxin/Cytokinin induces callus

Indirect organogenesis :
Advantages: Large number of plantlets may be produced

Disadvantage: the plantlets produced may not be true to type. The intervening callus phase may introduce genotypic and phenotypic variations/abnormalities

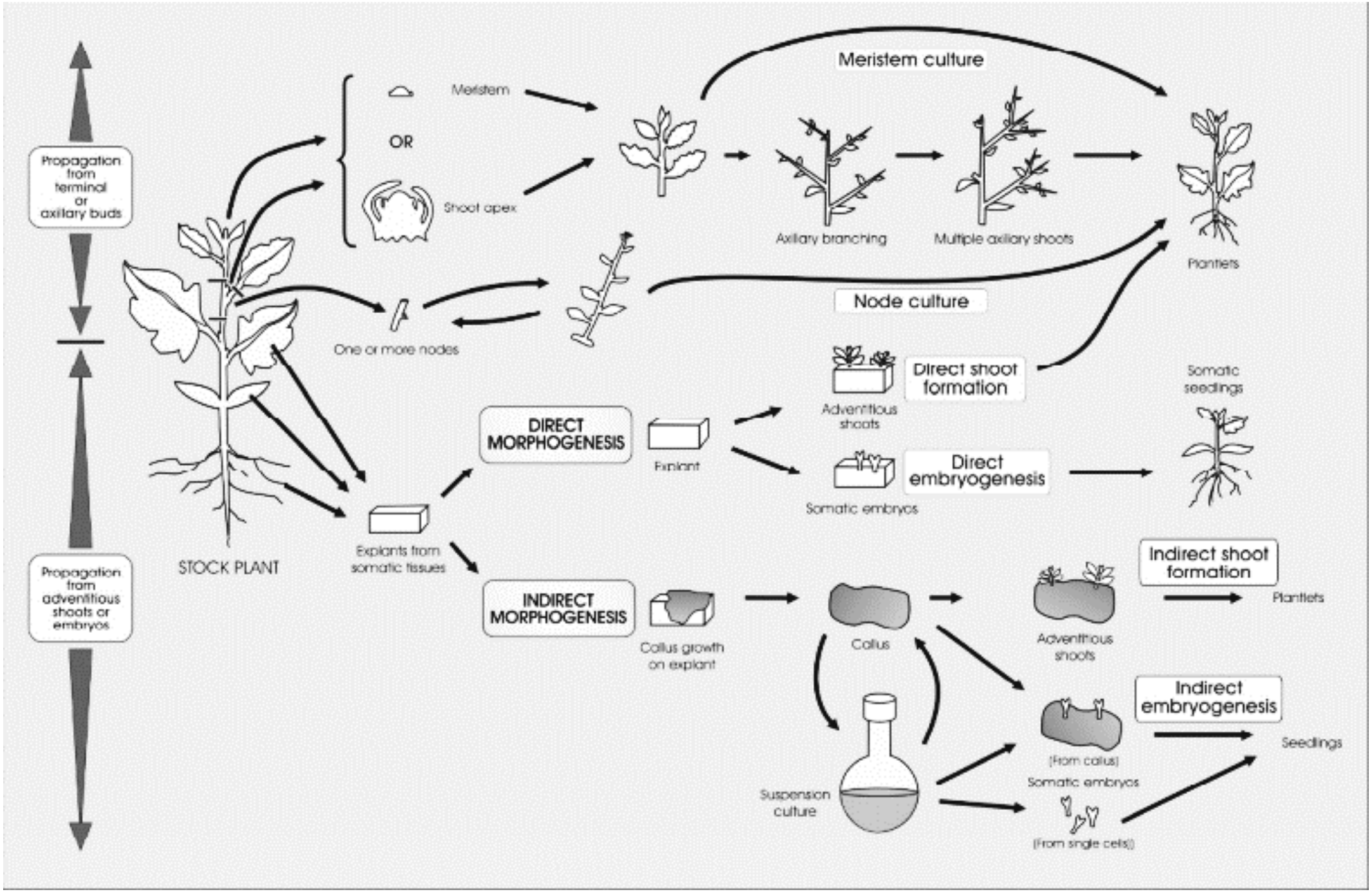
Somatic Embryogenesis:

Somatic Embryo: Also called as non zygotic embryo is an asexual embryo derived from a somatic cell

Somatic embryogenesis: In vitro formation of bipolar somatic embryos from asexual or somatic cell of plants.

Direct : The explants directly give rise to the somatic embryos

Indirect: The explants first dedifferentiate to form callus and from callus somatic embryos are formed


Propagation from terminal or axillary buds
Propagation from adventitious shoots or embryos
STOCK PLANT
Meristem
OR
Shoot apex
Meristem culture
Axillary branching
Multiple axillary shoots
Plantlets
One or more nodes
Node culture
Explants from somatic tissues
DIRECT MORPHOGENESIS
Explant
Adventitious shoots
Direct shoot formation
Somatic seedlings
Somatic embryos
Direct embryogenesis
INDIRECT MORPHOGENESIS
Callus growth on explant
Callus
Adventitious shoots
Indirect shoot formation
Plantlets
Suspension culture
(From callus)
Somatic embryos
Indirect embryogenesis
Seedlings
(From single cells)

Advantages of clonal propagation:

1. Small amount of explant required
2. Production and maintenance of large number of plants in limited space and time.
3. Rapid and cost effective clonal multiplication of desired varieties of plants
4. Multiplication of difficult to propagate plants
5. Is not effected by changing seasons, can be carried out all throughout the year
6. Beneficial in long term storage of germplasm
7. Prolonged storage of tissues for further use.
8. Disease elimination and production of certain virus free plants.

Disadvantages of Micropropagation:

1. Requirement of costly equipments, technologies, facilities and trained and skilled people
2. High capital investment and maintenance cost
3. Chances of contamination are high and pathogenic contamination can inflict heavy loss in short time
4. Genetic variability can be high in some culture types like callus culture
5. Repeated cycles of in vitro shoot proliferation may lead to a phenomenon called Vitrification or hyperhydricity

Vitrification/ Hyperhydricity is a physiological malformation that results in excessive hydration, low lignification, impaired stomatal function and reduced mechanical strength of tissue culture-generated plants.

Somaclonal Variation: Introduction

- The genetic and epigenetic changes observed in plants regenerated from cultured somatic cells are referred to as somaclonal variation (soma = vegetative, clone = identical copy)

- Term coined by Larkin and Scowcroft (1981).

- Other terms used to describe such variations are :
  - variation observed in the cultures of gametic cells is termed gametoclonal variation
  - calliclonal (somaclones from callus cultures)
  - protoclonal (somaclones from protoplast cultures)

- Depending on the final objective the variations may be desirable or undesirable:
  - for commercial micropropagation like clonal multiplication of valuable elite clones any kind of variation is worthless
  - Highly desirable if objective is inducing genetic variability

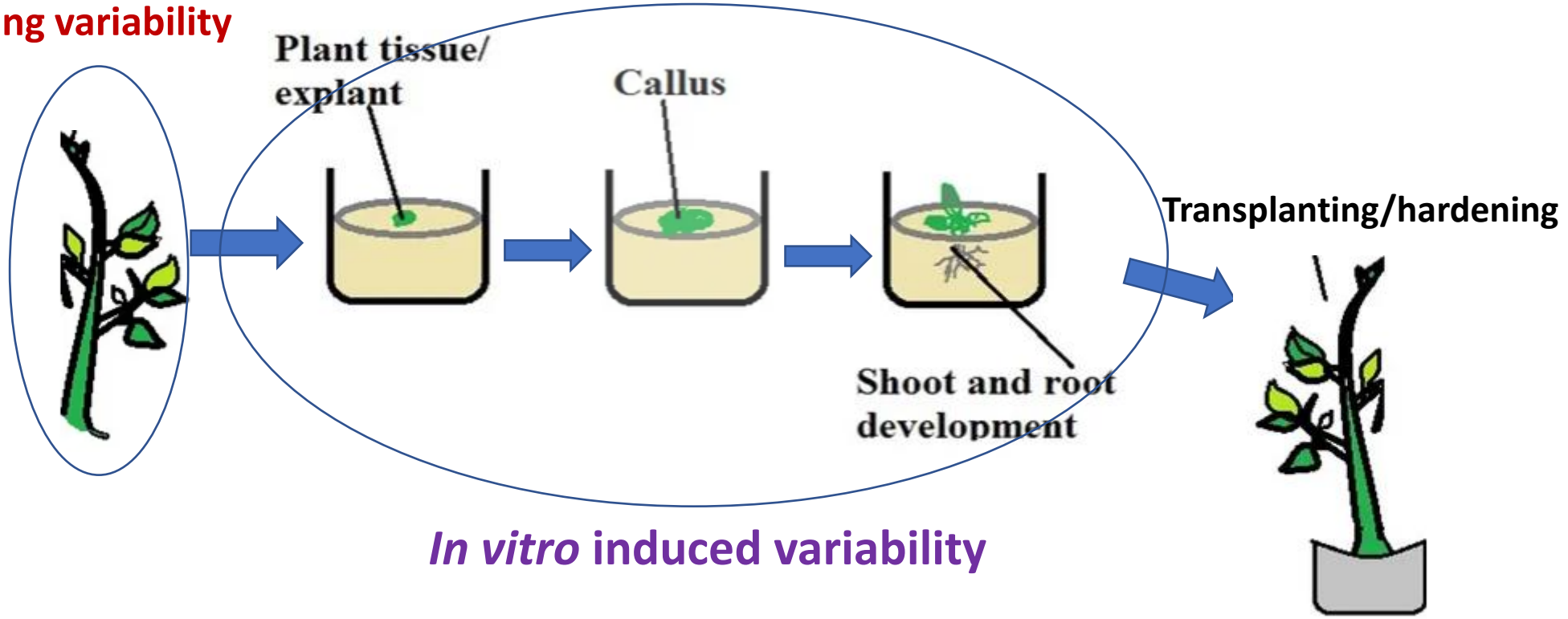
- Sugarcane is one of the plants in which the somaclonal variants were first observed in 1970s

- Somaclonal variations for karyotype, isozyme pattern, ploidy level, growth, yield, quality, pigmentation, disease resistance, and resistance to adverse soil and climatic conditions have all been reported in different plant species

Origin of somaclonal variation:

1) Pre existing variability
2) *Invitro* induced variability

Pre existing variability



***In vitro* induced variability**

Picture: Microscopemaster.com

1. Pre existing variability:

- Variation originating from the explant as a result of genetic heterogeneity of the cells of the initial explant

- extent of variation originating from the explant is dependent

  - **Age** : older the tissue higher the occurrence of genetic heterogeneity

  - **Type of tissue and organ**: Highly differentiated tissues such as roots, leaves, and stems generally produce more variations than explants with preexisting meristems, such as axillary buds and shoot tips

    - apical meristems (root tip and shoot tip), where cells divide by normal mitotic divisions, the cells are maintained at a uniform diploid level.
    - process of differentiation into specialized tissues may undergo duplication and endoreduplication to various degrees, resulting in somatic cells with 4C, 8C, or even higher levels of DNA.
      This phenomenon of polyploidization of body cells is termed **polysomaty.**

  - the older and/or the more specialized the tissue is used for regeneration, the greater the chances that variation will be recovered in the regenerated plants
  - **genotype of donor plant :**

- Somaclonal variation can also arise from somatic mutations already present in the donor plant, i.e., presence of chimera in explants

2.*Invitro* induced variability

Excision of tissue from the stable environment of the plant body and its transfer to culture medium under artificial conditions inside the culture vial could be a traumatic shock, causing a range of abnormalities, such as numerical and structural changes in the chromosomes, organization of DNA, mutations etc

Some of the factors known to induce somaclonal variation under *in vitro* conditions are:

1) Explant/explant source

- Highly differentiated tissues such as roots, leaves, and stems generally produce more variations than explants with preexisting meristems, such as axillary buds and shoot

- older the tissue is used for regeneration, the greater the chances that variation will be recovered in the regenerated plants

2) Mode of regeneration

- Both culture initiation and subsequent subculture expose explants to oxidative stress
- ‘extreme’ procedures such as protoplast culture and also callus formation impose stress
- the production of plants via axillary branching does not normally result in the production of variants, while cultures that go through a callus phase are the ones that theoretically promote a higher mutation rate
- more chromosome variability in the callus phase than in adventitious shoots

3) Effect of length of culture period and number of subculture cycles

- The longer a culture is maintained *in vitro*, the greater the somaclonal variation is
- Variant karyotypes are found to amass with increasing age of callus
- The frequency of variation increases with increase in the number of multiplication cycles, probably due to increased mutation rate with each cell cycles and/or accumulation of mutations over a period of time.

  Eg: after the eighth subculture, the number of somaclonal variants increased with a simultaneous decrease in the multiplication rate of propagules in banana
- Not only the number of subculture but their duration also contributes to enhancing the rate of somaclonal variations, especially cell suspension and callus cultures
- somaclonal variation is more apparent in plants regenerated from long-term cultures

4) Culture environment

- The culture medium, especially the growth regulators, is one of the various factors suspected to be the cause of somaclonal variation.

- In tissue cultures 2,4-D has proved highly mutagenic. 2,4-D either induces polyploidy or selectively favors division of polyploid cells.

5) Genotype and ploidy

- Among factors affecting somaclonal variation, plant genotype is probably the most important determinant of variation.

- Higher ploidy level plants show higher variation in regenerants

Mechanisms Underlying Somaclonal Variation:

1. **Changes in Chromosome Number and Structure:** Ploidy changes, including polyploidy, aneuploidy. Structural changes in chromosomes due to deletion (loss of genes), inversion (alteration of gene order), duplication (duplication of genes), and translocation (movement of chromosome segments to new location) could also bring about somaclonal variation without altering the chromosome number.

2. **Gene Mutations**: Several somaclonal variants due to recessive or dominant single or multiple gene mutations have been reported.

3. **Amplification of DNA:** Leading to enhancement of gene expression

4. **Hypomethylation of DNA:** DNA methylation leads to epigenetic changes

5. **Activation of Transposable Elements:** are mobile DNA segments that can move around in the genome and get inserted into coding regions and modify gene expression.

Advantages

- Cheaper than other methods of genetic manipulation.
- Tissue culture systems are available for many plant species.
- Not necessary to have identified the genetic basis of the trait, or indeed, in the case of transformation, to have isolated and cloned it.
- Novel variants have been reported among somaclones.
- Variation may be generated from different locations of the genome than those, which are accessible to conventional and mutation breeding.
- No possibility of obtaining chimeric expression if somaclones are raised through cell culture.

Disadvantage

- Inability to predict the outcome as they are random and lack reproducibility.
- The variations are usually negative.
- Positive changes are also altered in negative ways, sometimes.
- There are chances that the changes are not novel.
- The changes may not be stable after selfing or crossing.
- No *in vitro* selection methods exist for complicated traits such as yield, solids, sweetness, texture or shelf life

FIG 1

NUCLEAR GENE MUTATION OF TOMATO AFFECTING FRUIT COLOR. TOP, NORMAL TOMATO; CENTER, TANGERINE MUTATION; BOTTOM, YELLOW MUTATION.

FIG 5

BELL SWEET PEPPER VARIETY WITH LOW SEED NUMBER.

- Agriculture college Vellanikara: Ginger varieties released by somaclonal variation selection: Karthika, Athira, Aswathy; these are higher gingerol yielding varieties