

Plant Tissue Culture

for

M.Sc. Semester IV

(Paper 4)

Author

Dr. Subhash Kumar

Assistant Professor & HOD

Dept. of Botany, CCR (PG) College, Muzzaffarnagar (UP)

Dr. Seema Rani

Assistant Professor

Dept. of Botany, Meerut College, Meerut (UP)



Title: Plant Tissue Culture

Authors: Dr. Subhash Kumar and Dr. Seema Rani

ISBN: 978-93-49911-54-3

First Edition : 2026

Printing : Shiv Offset Printers, Meerut

DTP : Learning Media DTP Unit, Meerut

Design: Learning Media Designing Unit, Meerut

© ***Publisher***

Publisher: Learning Media Publication, Meerut

Regd. Office :

Learning Media Publication

A-16, Aman Vihar, Mawana Road,

Opp. J.P. Academy, Meerut- 250001

Contact No. +91-8449001390, 8791976106

E-mail : learningmediapublicationmeerut@gmail.com

Website : www.learningmedia.in

Price : ₹200

- The views expressed by the authors are their own. The editors and publishers do not own any legal responsibility or liability for the views of the authors, any omission or inadvertent errors.
- No part of this book may be reproduced or transmitted by any means/forms, electronic, mechanical, or any other way, without prior written permission from the publishers.
- Any dispute arising due to any issue/issues related to the publication of this book shall be subject to the jurisdiction of Meerut Courts only.

CONTENTS

UNIT 1. Plant Tissue Culture : An Introduction 1-25

Introduction to Plant Tissue Culture Introduction, terms and definitions, historical background, Laboratory organization, tools and techniques, Methods of sterilization, Basic techniques of plant tissue culture, Culture media: preparation and sterilization, Callus and suspension cultures

UNIT 2. Organ Culture and Protoplast Culture 26-47

Protoplast: Isolation, regeneration, and viability test, Somatic hybridization: Methods of protoplast fusion (chemical, viral, electrofusion), Practical applications of somatic hybridization, techniques and applications of somatic embryogenesis and plant regeneration, Anther, pollen, ovule, endosperm, and hairy root cultures

UNIT 3. Somaclonal Variation 48-71

Somaclonal variation: genetic basis and application in crop improvement, Cell/callus line selection for resistance to herbicides, stress, and diseases, Role of tissue culture in rapid clonal propagation, Production of pathogen-free plants and synthetic seeds

UNIT 4. Plant Transformation and Transgenic Crops 72-100

Methods of gene transfer in plants: Agrobacterium-mediated and CaMV-mediated gene transfer, Direct gene transfer: PEG, microinjection, electroporation, microprojectile (biolistics), liposome-mediated DNA delivery, Transgenic plants for crop improvement: Maize, Rice, Wheat, Cotton, Brinjal, and Tomato

UNIT 1

Plant Tissue Culture : An Introduction

PLANT TISSUE CULTURE

Plant Tissue Culture (PTC) is a foundational and revolutionary biotechnology technique that involves the aseptic in vitro culture of plant cells, tissues, or organs on a nutrient culture medium under controlled environmental conditions. At its core, PTC operates on two fundamental principles of plant biology: Totipotency (the inherent potential of a single plant cell to regenerate into a whole plant) and Plasticity (the ability of plants to alter their metabolism, growth, and development to best suit their environment). This technology allows for the clonal propagation of plants at an exponential rate, genetic manipulation, and the production of valuable metabolites, making it an indispensable tool in modern agriculture, horticulture, forestry, and pharmaceutical industries.

From Haberlandt's visionary failures to Steward's triumphant proof of totipotency and Murashige & Skoog's practical medium formulation, the history of Plant Tissue Culture is a testament to incremental scientific progress. Today, PTC is no longer just a laboratory curiosity; it is an essential pillar of global agriculture, enabling:

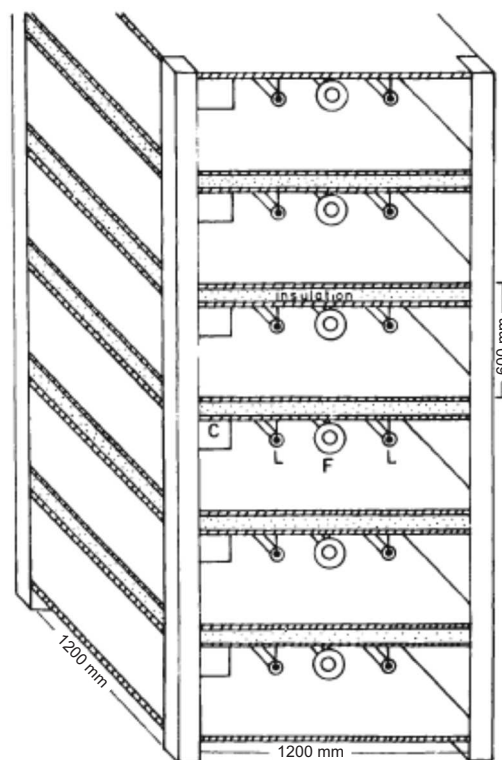


Diagram of a shelving unit especially designed for storing cultures. C, control panel; F, fan; L, light source.

- Mass propagation of elite, disease-free planting material (e.g., banana, potato, orchids, forest trees).



Illuminated shelves of culture racks with culture jars held in plastic trays

- Germplasm conservation via cryopreservation of endangered or non-seed producing species.
- Genetic improvement through somaclonal variation, haploid production, and as a prerequisite for genetic transformation.
- Production of high-value plant compounds (e.g., shikonin, paclitaxel) in controlled bioreactors.
- This technology perfectly bridges fundamental plant science with pressing real-world applications in food security, conservation, and medicine.

Key Terms and Definitions

- **Explant:** The initial piece of plant tissue (e.g., leaf segment, node, meristem, embryo) excised from a donor (mother) plant and used to initiate a culture.
- **Culture Medium:** A sterile, gel-like or liquid substance containing all the essential nutrients required for plant growth in vitro. Its key components are:
 - Macronutrients (N, P, K, Ca, Mg, S).
 - Micronutrients (Fe, Mn, Zn, B, Cu, Mo, Co).
 - Carbon Source (usually sucrose).

- Vitamins (e.g., myo-inositol, thiamine).
- Plant Growth Regulators (PGRs): The most critical components, which dictate the developmental pathway of the culture.
- **Auxins (e.g., 2,4-D, IAA, NAA):** Promote cell division and root initiation.
- **Cytokinins (e.g., BAP, Kinetin):** Stimulate shoot proliferation and cell division.
- **Gibberellins & Abscissic Acid:** Used in specific protocols for elongation or embryogenesis.
- **Callus:** An unorganized, proliferating mass of undifferentiated (parenchymatous) cells formed from an explant in response to injury and the presence of specific PGRs, particularly a high auxin-to-cytokinin ratio.
- **Totipotency:** The genetic potential of a single somatic plant cell to dedifferentiate, divide, and redifferentiate into a complete, fertile plant under appropriate conditions. This is the theoretical bedrock of all plant tissue culture.
- **Micropropagation:** The application of tissue culture techniques for the rapid, clonal multiplication of genetically identical plants.
- **Somatic Embryogenesis:** The process where somatic (non-reproductive) cells undergo a developmental pathway mimicking zygotic embryogenesis, leading to the formation of somatic embryos that can germinate into plantlets.
- **Organogenesis:** The de novo formation of plant organs (shoots or roots) from cultured tissues, either directly from the explant or indirectly via

an intervening callus phase. It is controlled by the ratio of auxins to cytokinins.

- **Somaclonal Variation:** Genetic or phenotypic variations observed in plants regenerated from tissue culture. It can be a source of undesirable off-types in clonal propagation or a valuable tool for crop improvement.
- **Protoplast:** A plant cell that has had its cell wall completely removed by enzymatic treatment (cellulase and pectinase), leaving a viable nucleus and cytoplasm surrounded only by the plasma membrane. Used for somatic hybridization.
- **Anther/Microspore Culture:** The culture of immature anthers or isolated microspores to produce haploid plants (with a single set of chromosomes), which can be subsequently doubled to create perfectly homozygous doubled-haploid lines, drastically speeding up plant breeding programs.

Historical Background

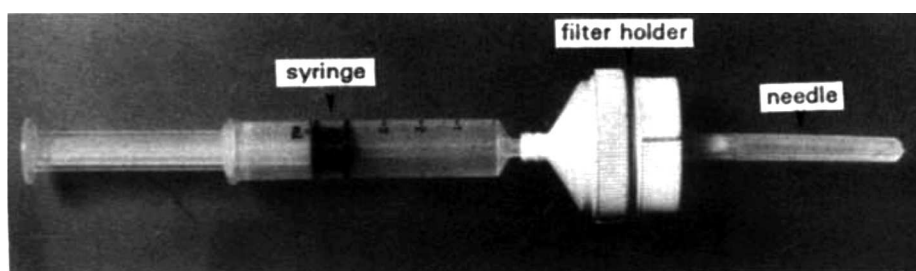
The development of plant tissue culture was a gradual process, built upon successive discoveries in plant physiology and aseptic technique over more than a century.

Period & Pioneer(s)	Key Contribution	Significance & Impact
1838-1839 Schleiden & Schwann	Proposed the Cell Theory, stating that the cell is the basic unit of life and that a complete organism can arise from a single cell.	Provided the philosophical foundation for totipotency, though it was not demonstrated for plants until much later.
1902 Gottlieb Haberlandt (Austria)	"Father of Plant Tissue Culture." Published his visionary experiments culturing isolated, fully differentiated leaf cells on a nutrient medium. He predicted totipotency but failed to achieve cell division due to the use of unsuitable PGRs (not yet discovered).	Laid out the concept and future potential of plant cell culture in his prophetic paper.
1930s-1940s Roger J. Gautheret (France), Philip R. White (USA), & Pierre Nobécourt (France)	Independently and successfully established continuously growing callus cultures of carrot (<i>Daucus carota</i>) and tobacco (<i>Nicotiana</i>). White's tomato root tip culture was the first perpetually growing organ culture.	Proved that plant tissues could be cultured indefinitely in vitro. This era marked the true beginning of practical plant tissue culture.
1957 F.C. Steward & K. Mears (USA)	Nurtured free-floating somatic cells from carrot phloem in a rotating liquid medium containing coconut milk (a natural source of cytokinins). These cells developed into whole carrot plants, providing the first definitive experimental proof of Totipotency.	A landmark achievement that validated Haberlandt's hypothesis and opened the door to mass plant regeneration.

1960 G. Morel (France)	Developed the technique for shoot-tip (meristem) culture to produce virus-free orchids (Cymbidium) and for their rapid clonal propagation.	Revolutionized the floriculture industry and introduced micropropagation and virus elimination as major commercial applications.
1962 Toshio Murashige & Folke Skoog (USA)	Formulated the most famous and widely used plant tissue culture medium—the Murashige and Skoog (MS) Medium—with optimized salt concentrations for tobacco callus culture.	MS medium became the universal standard, dramatically improving growth and consistency across countless plant species.
1964 S. Guha & S.C. Maheshwari (India)	Successfully produced haploid plants from cultured anthers of <i>Datura innoxia</i> .	Pioneered androgenesis, providing plant breeders with a powerful tool to create homozygous lines rapidly.
1970s Onwards	Advent of protoplast fusion (somatic hybridization), genetic transformation using <i>Agrobacterium</i> , and large-scale bioreactor culture for secondary metabolite production.	Transitioned tissue culture from a research tool to a core technology for genetic engineering, synthetic seed production, and industrial phytochemistry.

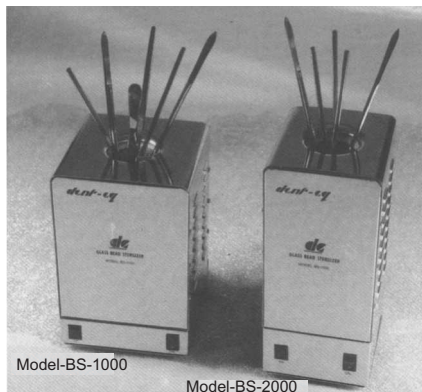
LABORATORY ORGANIZATION, TOOLS AND TECHNIQUES

A Plant Tissue Culture (PTC) laboratory is a highly specialized environment designed to maintain absolute aseptic (sterile) conditions to prevent microbial contamination, which is the single greatest cause of failure in culture work. Its organization is a careful orchestration of space, equipment, and procedure to ensure the successful initiation and maintenance of *in vitro* cultures. The workflow is linear and unidirectional to prevent cross-contamination, moving from a "dirty" area to a "clean" one.



'Swinnex' Millipore filter assembly for sterilizing small volumes of liquids. The needle is not always required.

The success of a plant tissue culture laboratory hinges on its disciplined organization and the rigorous, unwavering application of aseptic techniques. From the unidirectional flow of materials to the precise chemical sterilization of a delicate explant, each step is designed to create and maintain a pristine environment where totipotent plant cells can be guided to



Glass bead sterilizers

regenerate into whole organisms, free from the competitive pressures of the microbial world.

Laboratory Organization

A standard PTC lab is divided into distinct functional zones, often in separate rooms or physically partitioned spaces.

1. Washing and Storage Area:

- **Location:** The entry point of the workflow.
- **Function:** Cleaning of all glassware, tools, and containers. Storage of clean and sterilized items.
- **Key Equipment:** Large sinks, detergent baths, drying racks, autoclave, storage cabinets.

2. Media Preparation Room:

- **Location:** Adjacent to the washing area.
- **Function:** Weighing, mixing, and pH adjustment of culture media. The media is dispensed into culture vessels here.
- **Key Equipment:** Analytical and top-loading balances, pH meter, magnetic stirrers/hot plates, distilled/deionized water system,

microwave or hot plate for gelling agent (agar), dispensing pumps or pipettes, shelves for chemicals and stock solutions.

3. Sterilization Room:

- **Location:** Near the media prep room.
- **Function:** Dedicated space for sterilizing media, tools, and other supplies.
- **Key Equipment:** Autoclave (for steam sterilization under pressure at 121°C for 15-20 mins), membrane filter units for heat-labile compounds.

4. Inoculation/Transfer Room:

- **Location:** The core sterile area. Often an enclosed room with restricted access.
- **Function:** The aseptic transfer of explants to culture media. This is the most critical area for contamination control.

Key Equipment & Features:

- **Laminar Air Flow Hood (LAFH):** The centerpiece. It provides a horizontal or



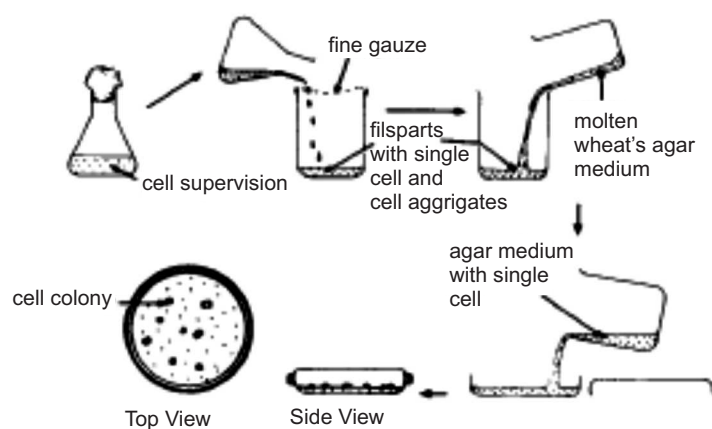
Laminar air-flow cabinets

vertical stream of HEPA-filtered, sterile air over the work surface, creating a particle-free environment for manipulations.

- **Germicidal UV Lamp:** Mounted inside the LAFH to sterilize the interior surface when not in use.
- **Alcohol Burner or Bunsen Burner:** For flame-sterilizing instruments.
- **Sterile Work Surface:** A smooth, non-porous surface (often stainless steel) that can be wiped with disinfectant (e.g., 70% ethanol).

5. Culture Room / Growth Room:

- **Location:** After the inoculation room.
- **Function:** Incubation of cultures under controlled environmental conditions.
- **Key Features:** Controlled Temperature (typically $25\pm 2^{\circ}\text{C}$), Light (provided by cool white fluorescent or LED tubes with a 16-hr photoperiod), Humidity control, and shelving units to hold culture vessels.



Steps involved in Bergmann's technique of cell plating

Essential Tools and Techniques

Precision and sterility govern every tool and action in PTC.

Tool/Item	Image/Description	Primary Function in PTC	Sterilization Method
Culture Vessels	Glass or disposable plastic containers: Test tubes, Jam jars, Petri dishes, Magenta boxes.	To hold the sterile medium and the growing plant culture. Often have closures to allow gas exchange while preventing contamination.	Autoclaved (if reusable). Pre-sterilized disposables are used aseptically.
Dissecting Tools	Forceps (fine-tipped), Scalpels with disposable blades, Micro-Spatulas, Needles.	For precise excision, cutting, and transfer of explants and subculturing tissues.	Flame-Sterilized in the LAFH using a spirit lamp/burner, dipped in alcohol and flamed. Cooled before use.

Inoculation Aid	Glass Bead Sterilizer	An electric device that uses high-temperature (250°C) glass beads to sterilize tool tips within 10-20 seconds. Safer and more effective than repeated flaming.	Tools are inserted into the beads for a few seconds.
Measuring & Dispensing	Graduated Cylinders, Pipettes (micropipettes & serological), Dispensing Bottles.	For accurate measurement and transfer of liquids (media, sterilants, stock solutions).	Autoclaved (glass/plastic) or use of sterile, disposable tips/pipettes.
Sterile Containers	Sterile Petri Dishes, Beakers	Temporary holding of sterilized explants or tools during the dissection process within the LAFH.	Autoclaved before being brought into the LAFH.
Personal Protective Equipment (PPE)	Lab Coat, Latex/Nitrile Gloves, Face Mask, Hair Cap.	To minimize the introduction of human-borne contaminants (skin, hair, breath) into the sterile environment.	Gloved hands are regularly swabbed with 70% ethanol.

Key Techniques:

- **Aseptic Technique:** The overarching practice of working without introducing contaminants. This includes minimizing airflow disruption in the LAFH, working swiftly but carefully, and properly sterilizing all items entering the workspace.
- **Explant Preparation:** Meticulously trimming the donor tissue to the correct size and shape to maximize surface contact with the medium and removal of damaged cells.
- **Subculturing:** The periodic transfer of growing tissues (callus, shoots) to fresh media to provide new nutrients and space, essential for maintaining healthy, proliferating cultures.

METHODS OF STERILIZATION

Sterilization is the complete elimination or destruction of all forms of microbial life. The method chosen depends on the material being sterilized.

For Instruments, Glassware, and Culture Media

- **Autoclaving (Moist Heat Sterilization):**
 - **Principle:** Saturated steam under pressure. The standard condition is 121°C at 15 psi (pounds per square inch) for 15-20 minutes.
 - **Mechanism:** The high-pressure steam has a high latent heat of vaporization, which efficiently coagulates and denatures microbial proteins.

- **Used For:** All aqueous culture media, tools, glassware, and dry items that can withstand heat and moisture.
- **Limitation:** Cannot be used for heat-labile substances (e.g., some vitamins, plant growth regulators like Zeatin, antibiotics).

☞ For Heat-Labile Solutions

• Membrane Filtration:

- **Principle:** Physical removal of microorganisms by passing the liquid through a sterile membrane filter with a pore size of 0.22 μm or 0.45 μm , which is smaller than bacteria and fungal spores.
- **Apparatus:** Uses a syringe-driven filter unit or a vacuum-driven filtration assembly connected to a sterile receiving flask.
- **Used For:** Stock solutions of heat-sensitive PGRs, vitamins, amino acids, and enzymes.

☞ For Explant Surface Sterilization

This is a critical step, as the explant itself is the primary source of contamination. It involves a sequence of chemical treatments.

Sterilant	Common Concentration & Time	Mode of Action	Use & Precautions
Ethanol	70% (v/v) for 30-60 seconds	Denatures proteins and dissolves lipids. Acts as a wetting agent.	A quick dip pre-treatment to reduce surface tension and allow better penetration of subsequent sterilants. Not used alone.
Sodium Hypochlorite (NaOCl)	0.5% - 2.5% (v/v) for 5-15 mins (Commercial bleach is ~5% NaOCl).	Releases chlorine which is a strong oxidizing agent, disrupting cellular enzymes.	The most common and effective surface sterilant. Must be thoroughly rinsed off with sterile distilled water (3-5 times) as it is phytotoxic.
Mercuric Chloride (HgCl ₂)	0.1% (w/v) for 2-5 minutes	A heavy metal that causes protein precipitation.	Highly effective but extremely toxic to humans and the environment. Use is discouraged and often prohibited; replaced by safer alternatives.
Hydrogen Peroxide (H ₂ O ₂)	3-10% (v/v) for 5-10 mins	Oxidizes cellular components.	Useful for seeds with hard coats. Decomposes into water and oxygen, leaving no harmful residue.
Antibiotics	Variable; e.g., Streptomycin, Gentamicin	Inhibit bacterial protein synthesis or cell wall formation.	Sometimes added to the initial culture medium as a last resort for particularly stubborn endogenous bacterial contamination. Can affect plant growth.

Typical Explant Sterilization Protocol:

1. Wash explant thoroughly under running tap water.
2. Dip in 70% ethanol for 30-60 sec.
3. Treat with sodium hypochlorite solution (with a few drops of surfactant like Tween-20) for the optimal time.
4. Rinse 3-5 times with sterile distilled water in the LAFH.
5. Trim the edges (which may be damaged by the sterilant) before placing on culture medium.

BASIC TECHNIQUES OF PLANT TISSUE CULTURE

Plant Tissue Culture (PTC) encompasses a suite of core techniques that exploit the totipotency of plant cells. Each technique is designed for a specific purpose, from rapid clonal multiplication to genetic conservation and crop improvement. Mastering these foundational protocols is essential for any successful PTC operation.

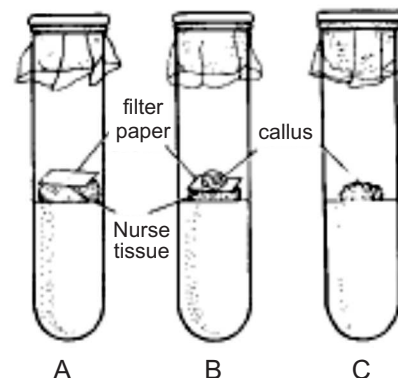
Micropropagation

- **Definition:** The aseptic, in vitro mass clonal multiplication of plants from small tissue explants.
- **Process:** Involves a defined sequence of stages on specific media formulations.
- **Applications:** Mass production of elite, disease-free planting material for horticulture (orchids, bananas), forestry (teak, eucalyptus), and agriculture (potato, strawberry).

Callus Culture

- **Definition:** The induction and maintenance of an unorganized, proliferating mass of cells (callus) from an explant.
- **Process:** Achieved by culturing explants on a medium with a high

auxin-to-cytokinin ratio (e.g., 2,4-D). Callus can be friable (soft, easily



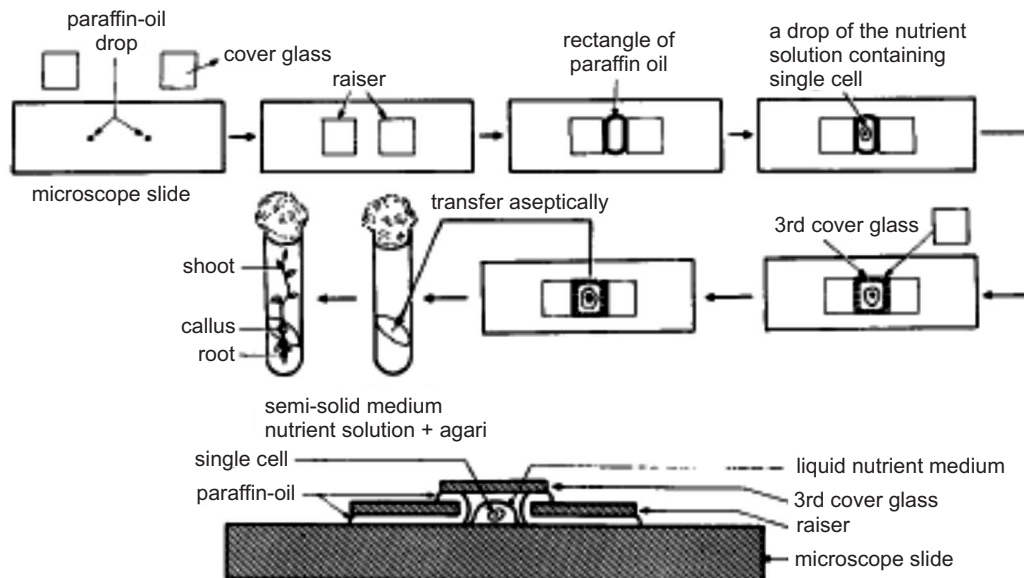
Nurse-tissue technique to raise single cell clones.

separated) or compact (hard).

- **Applications:** A prerequisite for somatic embryogenesis, suspension cultures, and protoplast isolation. Serves as a source of somaclonal variation for crop improvement.

Suspension Culture

- **Definition:** The culture of cells or small cell aggregates dispersed in a liquid nutrient medium kept in constant motion (on a shaker).



Diagrammatic summary of the steps involved in the microchamber technique of cell cloning

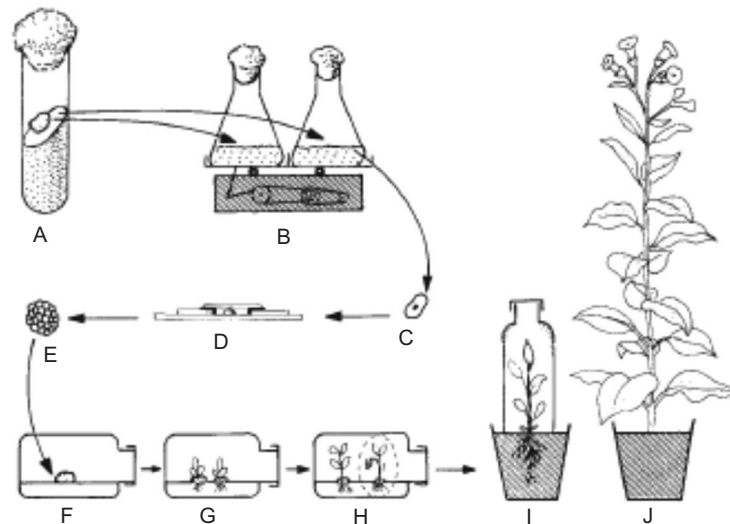
- **Process:** Initiated by transferring friable callus into liquid medium with constant agitation (100-120 rpm) for aeration.
- **Applications:** Ideal for large-scale production of secondary metabolites (e.g., paclitaxel from *Taxus*), for studies in cell physiology, and for protoplast isolation.

Organogenesis

- **Definition:** The de novo formation of plant organs (shoots or roots) from cultured tissues.
 - **Direct Organogenesis:** Organs form directly from the explant without an intervening callus phase.
 - **Indirect Organogenesis:** Organs form from a callus mass derived from the explant.
- **Process:** Controlled by the auxin:cytokinin ratio.
 - **High Cytokinin:** Auxin → Shoot Induction.
 - **High Auxin:** Cytokinin → Root Induction.
- **Applications:** The primary regeneration pathway in micropropagation and genetic transformation protocols.

Somatic Embryogenesis

- **Definition:** The process where somatic (non-reproductive) cells undergo developmental pathways to form bipolar structures (somatic embryos) possessing both a shoot and a root pole, analogous to zygotic embryos.
- **Process:** Involves two key stages:



Development of a tobacco plant from a single cell. A callus is raised from a small piece of tissue excised from the pith (A). By transferring it to a liquid medium and shaking the culture flasks (B) the callus is dissociated into single cells. A cell (C) is mechanically removed from the flask and placed in a drop of culture medium in micro-chamber (D). A small tissue (E) derived from the cell through repeated divisions is then transferred to a semi-solid medium where it grows into a large callus (F), and eventually differentiates plants (G,H). When transferred to soil (I,J) these plants grow to maturity, flower and set seeds (from the work of Vasil and Hildebrandt, 1965).

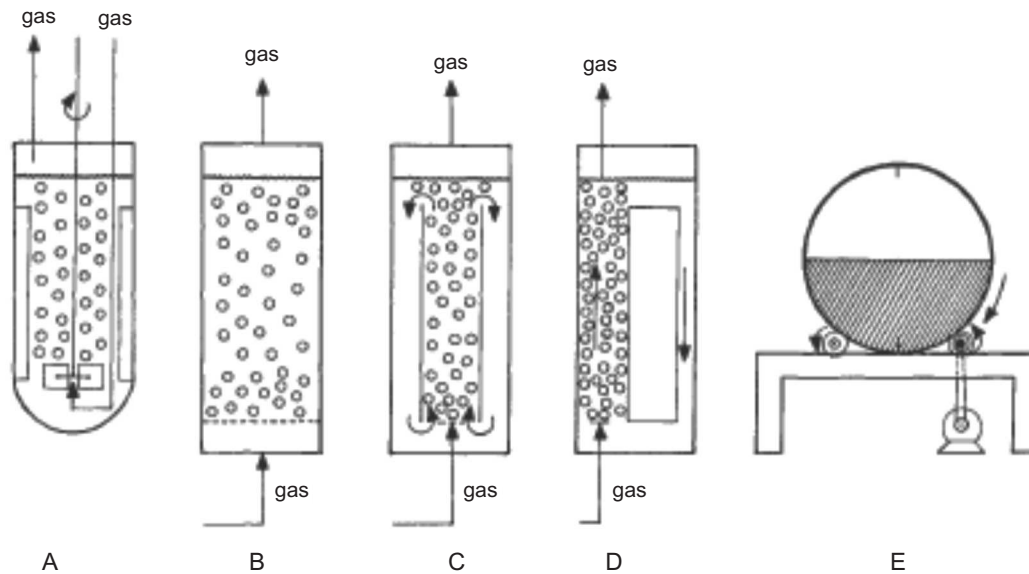
1. **Induction:** On a high-auxin (2,4-D) medium to form pro-embryogenic masses.
 2. **Maturation & Germination:** Transfer to a low-auxin or auxin-free medium for embryo development and conversion into plantlets.
- **Applications:** Enables mass clonal propagation via synthetic seeds, is highly efficient for large-scale automation, and is crucial for genetic transformation in many species.

BIOREACTORS USED IN PLANT TISSUE CULTURE

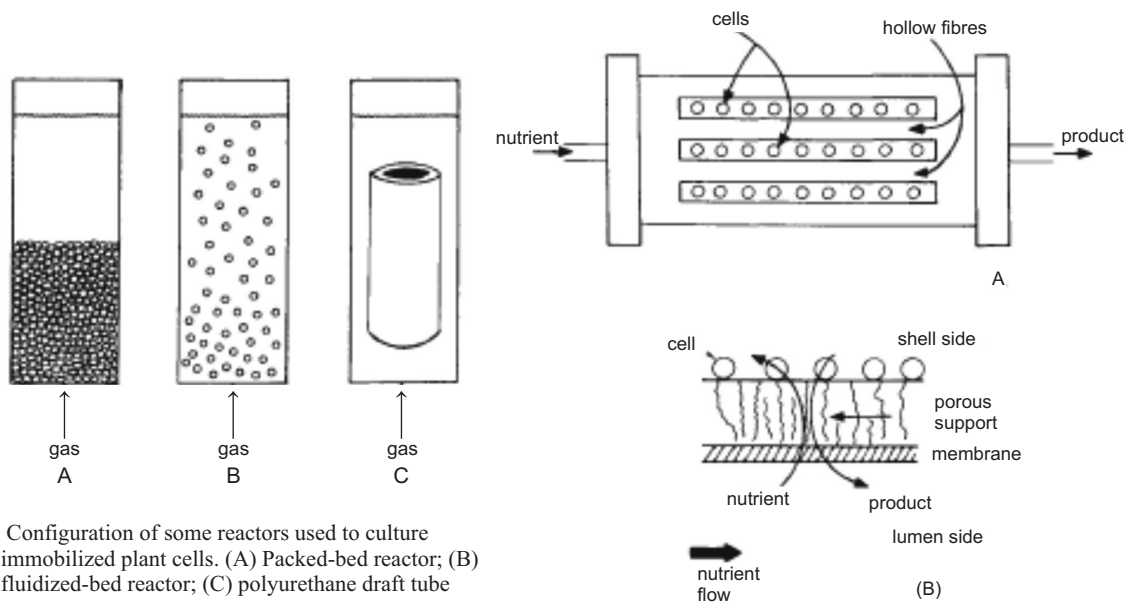
Plant tissue culture commonly utilizes specialized bioreactors designed to minimize shear stress while promoting growth, including Stirred-Tank, Airlift, Bubble Column, Temporary Immersion Systems (TIS), and Nutrient Mist bioreactors. These systems, often operating in batch or fed-batch modes, are crucial for large-scale micropropagation, hairy root culture, and production of secondary metabolites.

Key Types of Bioreactors in Plant Tissue Culture

Stirred-Tank Bioreactors (STBRs): Utilize mechanical impellers for mixing, though they are often modified with gentler agitation to prevent damaging sensitive plant cells.



Configuration of reactors used for plant cell cultivation. (A) Stirred-tank reactor; (B) bubble-column reactor; (C) air-lift reactor with draft-tube; (D) air-lift reactor with outer loop; (E) rotating-drum reactor.



Configuration of some reactors used to culture immobilized plant cells. (A) Packed-bed reactor; (B) fluidized-bed reactor; (C) polyurethane draft tube

(A) Hollow-fibre reactor for immobilized cell culture; (B) the portion marked in (A) enlarged to show the details of the reactor and the flow of nutrients and products across the membrane and the porous support of the hollow-fibre cartridge (adapted from Prenosil and Pederson, 1983).

Airlift Bioreactors

(ALBRs): Use air bubbles for both aeration and mixing (pneumatic agitation) rather than mechanical parts, reducing shear stress on cultures.

Bubble Column Bioreactors

Simple tanks where air is sparged from the bottom to provide oxygen and mix the culture.

Temporary Immersion Systems (TIS):

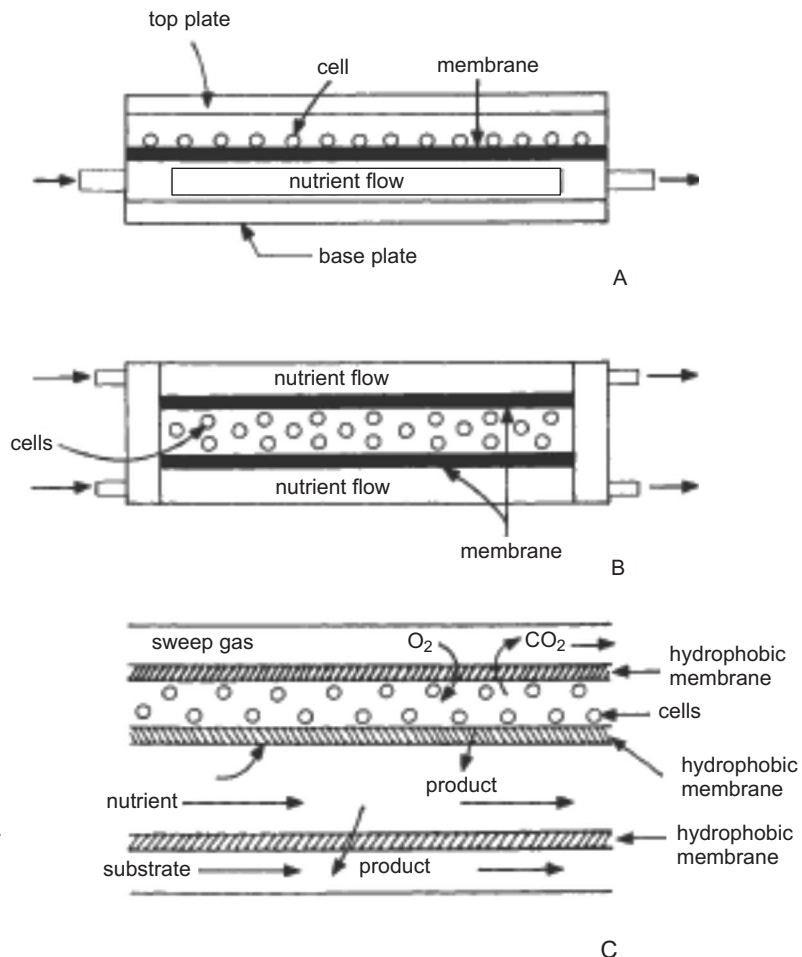
Examples like RITA® or twin-flask systems submerge plant tissues in liquid medium periodically, reducing hyperhydricity (vitrification) and providing better aeration.

Nutrient Mist/Spray Bioreactors:

Plants are grown on a support and nourished by a fine mist of nutrient medium, offering high oxygen availability.

Wave-Mixed/Disposable

Bioreactors: Utilize a rocking motion for gentle agitation and are favored for reducing contamination risks and cleaning needs.



Membrane reactor systems for immobilized plant cell culture.

(A) Flat plate membrane reactor with one side flow of nutrients; (B) flat plate membrane reactor with two side flow of nutrients; (C) multimembrane reactor system.

CULTURE MEDIA

The culture medium is the life-support system for in vitro plants. It must provide all essential nutrients, energy, and growth regulators.

The basic techniques of PTC are a toolkit for manipulating plant life at the cellular level. The success of any technique-be it micropropagation or somatic embryogenesis-is wholly dependent on the precise formulation and sterile preparation of the culture medium. This medium, a carefully balanced artificial ecosystem, provides the signals and sustenance that guide a single cell to realize its totipotent potential, forming the basis of a multi-billion dollar global biotechnology industry.

Key Components of a Standard Medium

1. **Macronutrients:** Supply major elements (N, P, K, Ca, Mg, S) in mM concentrations. Nitrogen is often provided as nitrate (NO_3^-) and ammonium (NH_4^+) salts.
2. **Micronutrients:** Trace elements (Fe, Mn, Zn, B, Cu, Mo, Co) required in μM concentrations. Iron is often chelated with Na_2EDTA to remain soluble at physiological pH.
3. **Carbon Source:** Sucrose (2-3%) is most common. It provides energy and osmotic balance, as photosynthesis in vitro is often limited.
4. **Vitamins:** Organic compounds essential for metabolism. Myo-inositol, Thiamine (B1), Nicotinic Acid (B3), Pyridoxine (B6) are standard.
5. **Plant Growth Regulators (PGRs):** The most critical variable. They dictate the developmental fate of the culture.
 - **Auxins (IAA, NAA, 2,4-D):** Promote cell division, callus formation, root initiation.
 - Cytokinins (BAP, Kinetin, Zeatin): Stimulate shoot proliferation, break apical dominance.
 - **Gibberellins (GA3):** Promote internode elongation and seed germination.
 - **Abscisic Acid (ABA):** Promotes somatic embryo maturation and induces dormancy.
6. **Gelling Agent:** Agar (0.7-1.0%) solidifies the medium to provide physical support. Gellan gum (Phytigel) is a clearer, purer alternative.
7. **pH:** Adjusted to 5.6-5.8 (slightly acidic) before autoclaving. This optimizes nutrient availability and agar solidification.

Common Media Types:

Medium Name (Inventor)	Key Characteristic	Primary Use
Murashige and Skoog (MS)	High salt concentration, especially nitrate and ammonium.	The universal standard for most dicot callus and shoot cultures.
Gamborg's B5	High nitrate but lower ammonium than MS.	Particularly good for suspension cultures of soybeans and other legumes.
White's (W)	Low salt concentration.	Originally for tomato root culture; now often used for anther culture and some established cultures.
Nitsch and Nitsch (NN)	Balanced macro and micronutrients.	Widely used for anther and microspore culture to induce haploids.
Woody Plant Medium (WPM)	Low ammonium, high nitrate, with specific micronutrients.	Formulated for woody species (trees, shrubs) which can be sensitive to high ammonium levels in MS.

Culture Media Preparation and Sterilization

This is a systematic, precise process where accuracy is paramount.

☞ Step-by-Step Preparation Protocol:

1. Stock Solution Preparation:

- To avoid weighing tiny amounts each time, concentrated stock solutions are prepared.
- Macronutrients (20X or 50X), Micronutrients (200X or 1000X), Iron (200X), Vitamins (100X or 200X), PGRs (1 mg/mL).
- Stocks are stored in labeled bottles at 4°C (PGR stocks at -20°C).

2. Media Compounding:

- **Water:** Measure ~80% of the final volume of distilled/demineralized water into a flask.
- **Additions:** While stirring on a magnetic stirrer, add stock solutions in the correct order to avoid precipitation (e.g., add iron last among salts).
- **Carbon Source:** Add weighed sucrose.
- **Gelling Agent:** Sprinkle agar while stirring to avoid clumping.
- **Volume:** Make up to the final volume with water.

3. pH Adjustment:

- Use a calibrated pH meter.
- Adjust to pH 5.6-5.8 using drops of 0.1N NaOH (to increase pH) or 0.1N HCl (to decrease pH).

4. Dispensing:

- Media is dispensed into culture vessels (tubes, jars) before sterilization.
- **For gelled media:** Dispense 15-25 mL per vessel.

- **For liquid media:** Dispense directly or after sterilization.

5. Sterilization - Autoclaving:

- Plug vessels with non-absorbent cotton, polypropylene closures, or lids.
- Load into an autoclave. The standard cycle is:
 - 121°C (250°F) at 15 psi (pounds per square inch) for 15-20 minutes.
 - **Important:** The timing starts once the temperature inside the chamber reaches 121°C, not from the beginning of the cycle.
- After the cycle, release pressure slowly to avoid boiling over. Let media cool and solidify on a clean, slanting surface if slants are required.

6. Adding Heat-Labile Compounds:

- Compounds destroyed by heat (e.g., certain antibiotics, some PGRs like Zeatin, enzymes) must be filter-sterilized.
- Using a 0.22 µm syringe filter, the compound is added to the cooled (40-50°C), but still liquid, autoclaved medium under aseptic conditions in a Laminar Air Flow Hood (LAFH) before it solidifies.

Quality Control & Storage:

- Prepared media should be clear and free of precipitates.
- Test for sterility by incubating 2-3 random vessels at 25°C for 3-5 days. Any cloudiness or fungal growth indicates contamination, and the entire batch should be discarded.
- Sterilized media can be stored at 4°C for 2-4 weeks to prevent drying and microbial growth.

CALLUS CULTURE

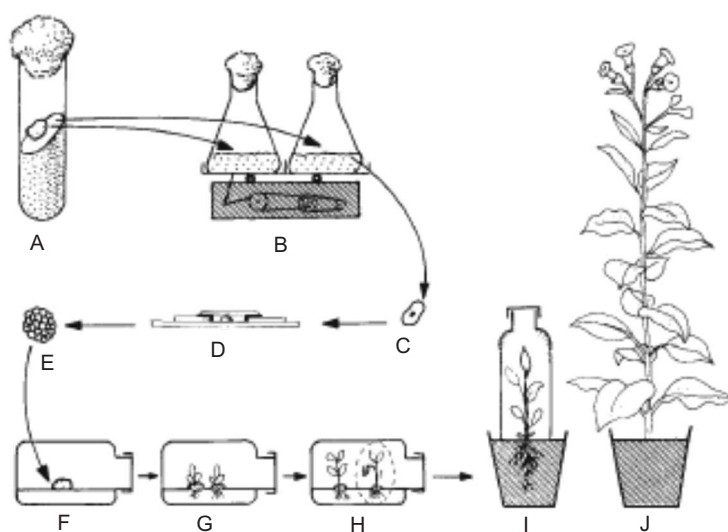
Callus culture is the essential gateway to unlocking the proliferative potential of plant cells *in vitro*, providing the raw cellular material for advanced applications. Suspension culture represents the refinement of this system, transforming static growth into a dynamic, scalable, and controllable process. Together, they form the indispensable cellular "factories" of plant biotechnology, enabling everything from fundamental research on totipotency to the industrial synthesis of life-saving drugs. The successful transition from a robust, friable callus to a fine, fast-growing suspension line is a key milestone in the development of any plant-based bioprocess.

Definition and Concept

A callus is an unorganized, proliferating mass of predominantly parenchymatous cells that forms from plant tissues (explant) in response to wounding and the presence of specific hormonal cues *in vitro*. It is characterized by the loss of specialized tissue organization, with cells reverting to a meristematic, dedifferentiated state. Callus formation is the primary manifestation of cellular totipotency in culture.

Process of Callus Induction and Maintenance

1. **Explant Selection:** The choice of explant is critical. Young, meristematic



Development of a tobacco plant from a single cell. A callus is raised from a small piece of tissue excised from the pith (A). By transferring it to a liquid medium and shaking the culture flasks (B) the callus is dissociated into single cells. A cell (C) is mechanically removed from the flask and placed in a drop of culture medium in micro-chamber (D). A small tissue (E) derived from the cell through repeated divisions is then transferred to a semi-solid medium where it grows into a large callus (F), and eventually differentiates plants (G,H). When transferred to soil (I,J) these plants grow to maturity, flower and set seeds

tissues (e.g., leaf segments, hypocotyls, cambium) are more responsive than mature, highly differentiated tissues.

2. **Culture Medium:** A medium rich in auxins, particularly 2,4-Dichlorophenoxyacetic acid (2,4-D), is essential. The standard protocol uses a high auxin-to-cytokinin ratio.

- MS or B5 medium supplemented with 2,4-D (1-3 mg/L) and sometimes a low concentration of cytokinin (e.g., BAP 0.1-0.5 mg/L).

3. **Induction Phase:** The explant swells, and within 2-4 weeks, cells at the cut surface begin to divide, forming a visible, soft mass-the primary callus.

4. **Subculturing:** The primary callus is fragmented (subcultured) onto fresh medium of similar composition every 4-6 weeks to maintain vigorous growth. This leads to the establishment of a callus line.

5. Callus Types:

- **Friable Callus:** Soft, breaks apart easily into single cells and small aggregates. Ideal for initiating suspension cultures.
- **Compact Callus:** Hard, dense, and organized; difficult to disaggregate.

Applications of Callus Culture

- **Prerequisite for Other Techniques:** Essential first step for somatic

embryogenesis, suspension cultures, and protoplast isolation.

- **Source of Somaclonal Variation:** Genetic variation arising in callus cells can be a tool for crop improvement (e.g., selecting disease-resistant or stress-tolerant lines from regenerated plants).
- **Metabolite Production:** Some calli can produce valuable secondary metabolites (e.g., shikonin from *Lithospermum* callus).
- **Genetic Transformation:** Callus is a common target tissue for *Agrobacterium*-mediated or biolistic transformation.

Key Factors Influencing Callus Culture

Factor	Influence on Callus Formation
Explant Source	Juvenile tissue > mature tissue; dicots > monocots.
Genotype	High genetic variability in response; some species/cultivars are recalcitrant.
Growth Regulators	2,4-D is most effective for induction. Type and concentration are species-specific.
Physical Conditions	Dark incubation often promotes callus growth over organogenesis.

SUSPENSION CULTURE

Definition and Concept

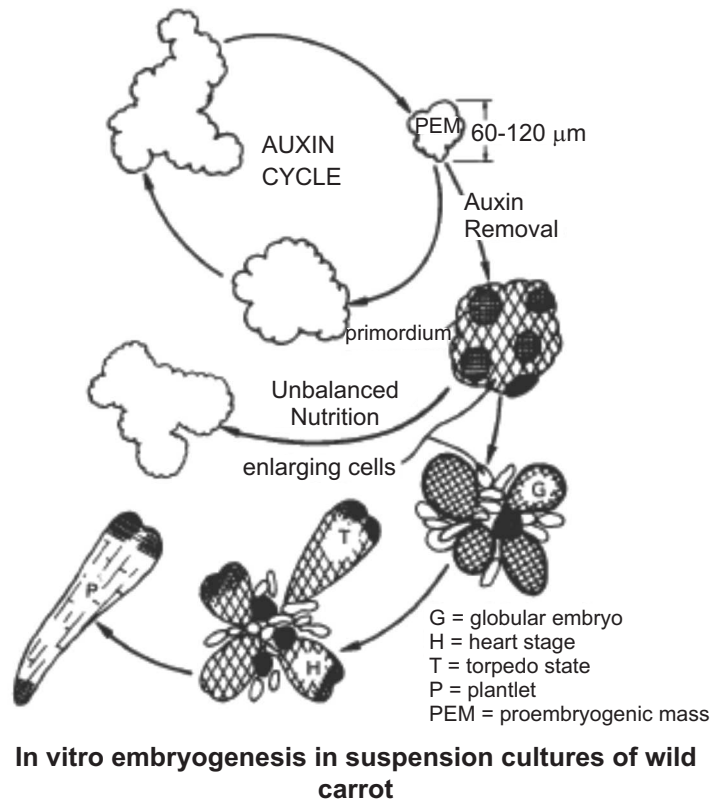
Suspension culture involves growing single plant cells and small cell aggregates dispersed in a constantly agitated liquid

nutrient medium. This system creates a more homogeneous and controlled environment than solid callus, allowing for

precise studies of cell physiology, nutrition, and large-scale production.

Process of Initiating and Maintaining Suspension Cultures

1. **Inoculum Source:** Initiated by transferring friable callus (approx. 2-3 grams Fresh Weight) into a flask containing liquid medium.
2. **Culture Medium:** Similar to callus medium but often with adjusted PGRs and reduced or omitted gelling agent. B5 medium is frequently preferred for its lower ammonium content.
3. **Agitation:** The flask is placed on a gyratory shaker at 100-150 rpm. Agitation serves three key purposes:
 - Aeration: Ensures adequate oxygen supply.
 - Cell Separation: Prevents aggregation and breaks up large clumps.
 - Mixing: Maintains uniform distribution of cells and nutrients.
4. **Growth Cycle:** Suspension cultures follow a standard microbial-like growth curve:
 - **Lag Phase:** Cells adjust to the liquid environment.
 - **Exponential (Log) Phase:** Rapid cell division.
 - **Stationary Phase:** Growth plateaus as nutrients deplete/waste accumulates.
 - **Decline Phase:** Cell death occurs.
5. **Subculturing:** Performed during the late exponential or early stationary phase (usually every 7-14 days) by transferring a small aliquot (e.g., 10-20% v/v) of the old culture into fresh medium. This maintains the culture in a state of continuous growth.



Types of Suspension Cultures

- **Batch Culture:** Cells grow in a fixed volume of medium until growth ceases (closed system). Growth is limited and follows a sigmoid curve.
- **Continuous Culture:** Fresh medium is continuously added, and an equal volume of spent culture is removed, maintaining cells in constant, steady-state growth (open system). Includes:

- **Chemostat:** Nutrient-limited steady state.
- **Turbidostat:** Cell density-limited steady state.

Applications of Suspension Culture

- **Large-Scale Production of Secondary Metabolites:** The only feasible method for industrial production of plant-derived pharmaceuticals in bioreactors.
- **Example:** Paclitaxel (Taxol) from *Taxus* sp. cell suspensions.
- **Protoplast Isolation:** Suspensions provide an excellent source of healthy, dividing cells for enzymatic cell wall removal.
- **Studies in Cell Physiology:** Ideal for investigating nutrient uptake, metabolic pathways, and the effects of toxins/elicitors in a controlled system.
- **Somatic Embryogenesis:** Many species produce somatic embryos efficiently in liquid medium, enabling automation.

Key Parameters in Suspension Culture

Parameter	Importance & Monitoring
Inoculum Density	Critical for revival; too low leads to lag or death, too high causes early nutrient depletion.
Growth Measurement	Packed Cell Volume (PCV), Fresh Weight (FW), Dry Weight (DW), Cell Count (using haemocytometer).
Aggregation	Small aggregates (10-20 cells) are desirable; large clumps limit nutrient diffusion and cause heterogeneity.
Shear Stress	High agitation speeds can damage cells; optimized for each cell line.

Comparison Table: Callus vs. Suspension Culture

Feature	Callus Culture	Suspension Culture
Physical State	Solid/Gelled medium (Agar).	Liquid medium.
Growth Pattern	Heterogeneous; cells grow as a surface mass.	More homogeneous; cells dispersed throughout medium.
Oxygenation	Limited by diffusion through the agar and callus mass.	Efficient through agitation and aeration.
Cell-to-Cell Contact	High, forming a tissue-like mass.	Low, primarily single cells and small aggregates.
Scale-Up Potential	Low; limited by surface area.	High; can be scaled up in bioreactors (100L to 20,000L).
Subculture Method	Manual fragmentation and transfer.	Aseptic pipetting or pumping of an aliquot.
Primary Applications	Regeneration studies, transformation target, metabolite studies.	Large-scale metabolite production, protoplast isolation, physiological studies.
Monitoring Growth	Visual assessment, fresh/dry weight of entire mass.	PCV, cell counting, turbidity, fresh/dry weight of sampled volume.

QUESTIONS



Section A: Very Short Answer Type Questions

1. Define explant.

Ans. An explant is a piece of plant tissue or organ taken from a donor plant and used to initiate a culture in vitro.

2. What is the full form of MS media?

Ans. Murashige and Skoog media.

3. Name two chelating agents used in plant tissue culture media.

Ans. EDTA (Ethylenediaminetetraacetic acid) or Sodium EDTA.

4. What is subculturing?

Ans. The process of transferring cells, tissues, or organs from an existing culture to a fresh medium to sustain growth.

5. Define totipotency.

Ans. The inherent capacity of a single plant cell to divide, differentiate, and develop into a complete plant.

6. What is the purpose of adding agar to the culture medium?

Ans. Agar acts as a gelling agent to provide a solid or semi-solid support matrix for explant growth.

7. Name a common plant growth regulator used for inducing callus.

Ans. 2,4-Dichlorophenoxyacetic acid (2,4-D).

8. What is the standard temperature for maintaining plant tissue cultures?

Ans. $25 \pm 2^{\circ}\text{C}$.

9. Give one example of a phenolic exudation inhibitor used in media.

Ans. Polyvinylpyrrolidone (PVP) or Activated Charcoal.

10. What is meant by aseptic technique?

Ans. A set of procedures performed under sterile conditions to prevent contamination by microorganisms.

Section B: Short Answer Type Questions

11. Briefly describe the historical contribution of Gottlieb Haberlandt to plant tissue culture.

Ans. Gottlieb Haberlandt is recognized as the "Father of Plant Tissue Culture." In 1902, he proposed the concept of totipotency and made the first attempt to culture isolated plant cells on

a nutrient medium. Although his cells did not divide, his visionary ideas laid the foundational principles for the entire field.

12. Differentiate between a laminar air flow cabinet and an autoclave based on their purpose in a PTC lab.

Ans. A Laminar Air Flow (LAF) cabinet provides a sterile, particulate-free workspace (aseptic environment) for conducting procedures like inoculation and subculturing. An autoclave, on the other hand, is used for sterilization. It uses pressurized steam (at ~121°C and 15 psi) to sterilize culture media, tools, glassware, and liquids by killing all microorganisms.

13. Why are macronutrients required in relatively high concentration in culture media?

Ans. Macronutrients (N, P, K, Ca, Mg, S) are the essential building blocks of major plant biomolecules like proteins, nucleic acids, chlorophyll, and cell walls. They are required in amounts >0.5 mM/L to support fundamental processes such as cell division, elongation, metabolism, and maintaining osmotic potential.

14. List four basic components of a synthetic culture medium.

Ans. The four basic components are:

- (i) Inorganic Nutrients (Macro & Micro-elements),
- (ii) Organic Supplements (Vitamins, amino acids),
- (iii) Carbon Source (Sucrose),
- (iv) Gelling Agent (Agar-agar for solid media).

15. What is callus? Mention one of its characteristic features.

Ans. Callus is an unorganized, undifferentiated, and proliferating mass of parenchymatous cells formed in vitro from an explant in response to plant growth regulators. A key characteristic is its genetic heterogeneity, leading to somaclonal variation.

16. Explain the term "suspension culture."

Ans. Suspension culture involves growing single cells or small aggregates of cells dispersed in a liquid nutrient medium, which is agitated continuously. The agitation ensures proper aeration, mixing of nutrients, and breakdown of cell clumps. It is used for large-scale propagation, metabolite production, and studies in cell physiology.

17. Why is sterilization of culture media essential? Describe one method.

Ans. Sterilization is essential to eliminate all contaminating microorganisms (bacteria, fungi) that would otherwise outcompete and kill the plant tissues. The standard method is autoclaving at 121°C under 15 psi pressure for 15-20 minutes, which denatures microbial proteins and enzymes.

18. What is the role of cytokinins in culture media?

Ans. Cytokinins (e.g., BAP, Kinetin) primarily promote cell division (cytokinesis). They help in breaking apical dominance, stimulating shoot proliferation, and inducing adventitious bud formation. In conjunction with auxins, they regulate morphogenesis (organogenesis).

19. Write a short note on the importance of pH in media preparation.

Ans. The pH (typically adjusted to 5.6-5.8) is critical for multiple reasons: it affects the solubility of salts, the stability of media components (e.g., agar gel strength), the activity of plant growth regulators, and the uptake of nutrients by plant cells. Incorrect pH can inhibit growth or cause nutrient deficiencies.

20. Distinguish between totipotency and plasticity.

Ans. Totipotency is the specific potential of a single cell to give rise to a whole organism. Plasticity is a broader characteristic of plant cells, allowing them to alter their metabolism, growth, and development in response to environmental conditions or stimuli, which is the basis for dedifferentiation and redifferentiation in culture.

Section C: Long Answer Type Questions

21. Describe in detail the organization and essential facilities of a standard Plant Tissue Culture laboratory.

Ans. A standard PTC lab is designed to maintain aseptic conditions and is organized into a sequence of functional areas to ensure a unidirectional workflow and prevent contamination.

- **Media Preparation Room:** Equipped with balances, pH meter, stirrers/hot plates, distilled/deionized water source, microwave/autoclave, and storage shelves for chemicals, glassware (flasks, pipettes), and culture vessels.
- **Washing and Storage Area:** Dedicated space with sinks, detergents, drying racks/ovens, and storage for cleaned items.
- **Sterilization Room:** Houses the autoclave for sterilizing media, tools, and glassware. May also have a dry heat oven for heat-stable items.
- **Aseptic Transfer Area:** The core area containing Laminar Air Flow (LAF) cabinets. The LAF provides a HEPA-filtered, sterile horizontal air flow for performing all inoculations and subcultures. A spirit lamp, forceps, scalpels, and a dissecting microscope are kept here.
- **Culture Room/Incubation Room:** An environmentally controlled room with temperature ($25 \pm 2^\circ\text{C}$), humidity, and light (provided by cool white fluorescent tubes with a photoperiod control) regulation. Cultures are maintained on racks or shelves.
- **Observation/Data Collection Area:** A space with microscopes and other instruments for monitoring and analyzing culture growth.

Proper lab organization with separate areas for dirty and clean processes, along with disciplined personnel hygiene, is critical to the success of tissue culture operations.

22. Elaborate on the steps involved in the preparation and sterilization of a solid culture medium (e.g., MS Medium).

Ans. The preparation involves a systematic sequence:

1. **Calculation and Weighing:** Precisely weigh all stock solutions or individual chemicals as per the MS medium formulation.
2. **Dissolution:** Add about 2/3rd of the final volume of distilled water to a beaker/flask. Dissolve macro-elements, micro-elements, iron source (Fe-EDTA), and organic

components (vitamins, myo-inositol) sequentially with constant stirring. Heat may be applied carefully for dissolution.

3. **Addition of Carbon Source:** Add the required amount of sucrose (typically 2-3%) and stir until completely dissolved.
4. **pH Adjustment:** Adjust the pH of the medium to 5.8 using 0.1N NaOH or 0.1N HCl with a calibrated pH meter. This is crucial for nutrient availability and agar solidification.
5. **Addition of Gelling Agent:** Add the powdered agar (0.7-1.0%) and heat the mixture with constant stirring until the agar completely melts and the solution becomes clear.
6. **Dispensing:** While still hot and liquid, dispense the medium into suitable culture vessels (flasks, jars, tubes) using a dispenser or funnel, avoiding spillage on rims.
7. **Closure:** Plug or cap the vessels loosely to allow steam penetration during sterilization.
8. **Sterilization:** Autoclave the media at 121°C (15 psi) for 15-20 minutes. For heat-labile components (e.g., some hormones, antibiotics), filter-sterilize using a 0.22 µm membrane filter and add to the cooled, autoclaved medium under aseptic conditions.
9. **Cooling and Storage:** Allow the medium to cool and solidify in a clean area. Prepared media can be stored for a short period at 4°C to prevent dehydration.

23. What is callus culture? Describe the protocol for its induction from a leaf explant, including the role of growth regulators.

Ans. Callus Culture is the in vitro proliferation of unorganized, undifferentiated parenchymatous cells from an explant.

Protocol for Induction from Leaf Explant:

1. **Selection & Sterilization of Explant:** Select a healthy, young leaf. Surface sterilize using a sequence of treatments: tap water wash → 70% ethanol dip (30-60 sec) → sodium hypochlorite (1-2%) or mercuric chloride (0.1%) treatment (2-5 min) → 3-5 rinses with sterile distilled water.
2. **Excision:** Under aseptic conditions in a LAF, cut the leaf into small segments (5-10 mm²), avoiding major veins.
3. **Inoculation:** Place the explant pieces with the abaxial (lower) surface in contact with the solid culture medium.
4. **Medium & Growth Regulators:** Use a basal medium like MS, supplemented with an auxin and often a cytokinin. For callus induction, a high auxin:cytokinin ratio is effective. 2,4-D (1-3 mg/L) is particularly potent for inducing callus from most dicot explants. Sometimes, a combination of 2,4-D and a low concentration of kinetin or BAP is used.
5. **Incubation:** Cultures are incubated in the dark or under low light at 25±2°C. Darkness often promotes callusing and prevents explant browning.
6. **Observation & Subculture:** Within 2-4 weeks, a creamy-white or pale green, friable or compact callus appears from the cut edges. This primary callus must be subcultured onto fresh medium of the same composition every 4-6 weeks to sustain growth.

Role of PGRs: Auxins (especially 2,4-D) trigger dedifferentiation of mature cells, re-initiate cell division, and promote unorganized growth. Cytokinins support cell division. Their balance determines the nature of the callus.

24. Compare and contrast solid (callus) culture and liquid (suspension) culture, highlighting their advantages, disadvantages, and applications.

Ans.

Feature	Solid (Callus) Culture	Liquid (Suspension) Culture
State of Medium	Solidified with agar.	Liquid, no gelling agent.
Growth Form	Undifferentiated cell mass attached to medium.	Single cells & small cell aggregates dispersed in medium.
Aeration	Poor, limited to surface.	Excellent due to agitation (shakers), ensuring uniform nutrient & gas exchange.
Nutrient Availability	Gradients can exist; cells may excrete waste locally.	Homogeneous; nutrients and wastes are evenly distributed.
Observation/Monitoring	Difficult to observe individual cells; growth measurement is indirect (fresh/dry weight).	Easy to sample and monitor cell growth (packed cell volume, cell counting).
Subculture	Manual transfer of callus pieces; can be slow.	Easy by pipetting a portion of the suspension; suitable for automation.
Scale-up Potential	Limited, labor-intensive.	High, can be scaled up to bioreactors for industrial production.
Applications	Initiation of cultures, organogenesis, somatic embryogenesis, conservation.	Large-scale clonal propagation, production of secondary metabolites, protoplast isolation, physiological studies.
Advantages	Simple setup, less prone to contamination spread, good for morphogenesis studies.	Higher growth rates, better control over environment, uniform biomass, suitable for biochemical studies.
Disadvantages	Heterogeneous cell types, nutrient gradients, slower growth, difficult for quantitative studies.	Requires specialized equipment (shakers), prone to shear stress, aggregation of cells can be a problem, higher risk of contamination spread.

25. Discuss the various methods employed for the sterilization of explants, instruments, and culture media in a plant tissue culture laboratory.

Ans. Sterilization is paramount and involves different methods for different materials:

A. For Explants (Surface Sterilization):

- **Chemical Sterilants: Ethanol (70%):** Quick surface wetting and killing of microbes; used for a brief dip (30-60 sec) before or after a stronger sterilant.
- **Sodium Hypochlorite (NaOCl, 0.5-2%):** Most common; effective with a surfactant (Tween-20). Treatment for 5-20 mins, followed by thorough rinsing.
- **Mercuric Chloride (HgCl₂, 0.1%):** Very potent but highly toxic; used for heavily contaminated explants for 2-5 mins, requiring extensive rinsing.

- **Hydrogen Peroxide (H_2O_2 , 3-10%):** Also used.
- **Antibiotics/Antifungals:** Sometimes added to the initial culture medium to control endogenous contamination.
- **Sequential Treatment:** A typical sequence is: Detergent wash → Running water rinse → 70% EtOH dip → NaOCl treatment → 3-5 sterile water rinses.

B. For Instruments and Glassware:

- **Autoclaving (Moist Heat):** For metal instruments (forceps, scalpels wrapped in aluminum foil), glassware, and plasticware that can withstand heat. 121°C, 15 psi for 20-30 mins.
- **Dry Heat Oven:** For glassware and metal instruments (e.g., scalpels). Sterilized at 160-180°C for 2-3 hours.
- **Flame Sterilization:** Direct flaming of instrument tips (using spirit lamp/Bunsen burner) in the LAF cabinet just before use. This is for instant sterilization during transfer operations.

C. For Culture Media and Heat-labile Solutions:

- **Autoclaving (Standard Method):** For all heat-stable media components and water. 121°C, 15 psi for 15-20 minutes.
- **Membrane Filtration:** For heat-labile substances like some plant growth regulators (GA3, ABA), vitamins, enzymes, and antibiotics. The solution is passed through a 0.22 μm porosity membrane filter under aseptic conditions into the sterile, cooled media.
- **Tyndallization:** A fractional sterilization method involving steaming at 100°C for 30 minutes on three consecutive days. Rarely used now, but an option for media components degraded by high autoclave temperatures.

The choice of method depends on the material's nature, its heat tolerance, and the level of sterility required.