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MATS CENTRE FOR DISTANCE & ONLINE EDUCATION

Plant Tissue Culture

**Bachelor of Science (B.Sc.)
Semester - 4**



SELF LEARNING MATERIAL

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PLANT TISSUE CULTURE
MATS University
PLANT TISSUE CULTURE
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MODULE INTRODUCTION

Course has five module . Under this theme we have covered the following topics:

Contents

MODULE I : INTRODUCTION TO PLANT TISSUE CULTURE
MODULE II: CALLUS CULTURE, CELL SUSPENSION CULTURE
MODULE III : INTRODUCTION TO HAPLOID PRODUCTION
MODULE IV: INTRODUCTION TO PROTOPLAST CULTURE
MODULE V: INTRODUCTION TO THE PRODUCTION OF
TRANSGENIC PLANTS

These themes of the Book discuss about Plant tissue culture is a technique that involves growing plant cells, tissues, or organs under sterile conditions on a nutrient medium, allowing for rapid propagation, disease-free plant production, and genetic resource preservation. This book is designed to help you think about the topic of the particular module. We suggest you do all the activities in the modules, even those which you find relatively easy. This will reinforce your earlier learning.



MODULE 1

INTRODUCTION TO PLANT TISSUE CULTURE

Objective:

To provide a comprehensive understanding of plant tissue culture, its types, aseptic

techniques, tissue culture media, and plant growth regulators.

Unit 1.1: Plant Tissue Culture

1.1.1 Introduction, Terms, and Definitions

Gottlieb Haberlandt, in 1902 tried to cultivate individual palisade cells from leaves in Knop's salt solution supplemented with sucrose. The cells sustained for a month stored starch but eventually did not divide. Despite his failure, he is considered the father of plant tissue culture since his experiment set the stage for developing tissue culture technology. Similarly, Roger J. Gautheret, a French scientist, had inspiring results with culturing cambial tissues of carrots in 1934.

Plant tissue culture is a collection of methods used to maintain or grow plant cells, tissues, or organs under sterile conditions on a nutrient culture medium of known composition. It is widely used to yield clones of a plant in a method known as micropropagation. Different techniques in plant tissue culture may offer certain advantages over traditional methods of propagation, including:

- The production of exact copies of plants that produce particularly good flowers, fruits, or other desirable traits.
- To quickly produce mature plants.
- To produce a large number of plants in a reduced space.
- The production of multiples of plants in the absence of seeds or necessary pollinators to produce seeds.
- The regeneration of whole plants from plant cells that have been genetically modified.

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- The production of plants in sterile containers allows them to be moved with greatly reduced chances of transmitting diseases, pests, and pathogens.
- The production of plants from seeds that otherwise have very low chances of germinating and growing, e.g., orchids and *Nepenthes*.
- To clean particular plants of viral and other infections and to quickly multiply these plants as 'cleaned stock' for horticulture and agriculture.
- Reproduce recalcitrant plants required for land restoration
- Storage of genetic plant material to safeguard native plant species.

Plant tissue culture is based on the ability of several plant parts to regenerate into a complete plant (cells of such regenerative plant parts are referred to as totipotent cells that can differentiate into many specialized cells). Individual cells, cell wall-less plant cells (protoplasts), fragments of leaves, stems or roots can usually be employed to produce a new plant on culture medium provided the necessary nutrients and plant hormones.



Fig: Typical plant tissue

1.1.2 Types of culture

Callus Culture



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A callus is an unorganized mass of cells that develops when cells are wounded. When the explant is cultivated on media that promote the development of undifferentiated cells, a callus is formed. The majority of callus cells are formed with the aid of auxins and cytokinins. Using plant growth hormones, callus can multiply continuously or be directed to develop organs or somatic embryos.

Cell Suspension Culture

Small fragments of loose friable callus can be cultured as cell suspension cultures in a liquid medium. Cell suspensions can be preserved as batch cultures grown in flasks for long periods. A portion of callus tissue can be transferred into a liquid medium, and when subjected to continuous shaking, single-cell cultures and suspension cultures can be cultivated from callus cultures. The growth rate of the suspension-cultured cells is generally advanced than that of the solid culture.

Anther/Microspore Culture

Anther or microspore culture is the culture of anthers or single microspores to generate haploid plants. Embryos may be generated through a callus stage or be a direct reiteration of the developmental states typical of zygotic embryos. Microspore culture, unlike classical breeding techniques, allows the generation of homozygous plants in a very short period. Such homozygous plants are valuable tools in plant breeding and genetic research.

Protoplast Culture

Protoplasts contain all the components of a [plant cell](#) except for the cell wall. Protoplasts can be used to create somatic hybrids and regenerate whole plants from a single cell. Cell walls of explant can be removed either mechanically or enzymatically. Protoplasts can be cultured either in liquid or solid medium. Protoplasts embedded in an alginate matrix and then cultured on a solid medium have better success rates of regeneration. Although protoplasts appear to be a very appealing



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method for regenerating plants and transferring genes, they are extremely delicate.

Embryo Culture

It is a technique in which isolated embryos from immature ovules or seeds are cultured in vitro. For species whose seeds are dormant, resistant, or prematurely sterile, embryo culture has been used as a helpful tool for direct regeneration. In plant breeding programs, embryo culture goes hand in hand with in vitro control of pollination and fertilization to ensure hybrid production. In addition, direct somatic embryos and embryogenic calluses can be produced from immature embryos.

Meristem Culture

Using apical meristem tips, it is possible to produce disease-free plants. This technique can be referred to as meristem culture, meristem tip culture, or shoot tip culture, depending on the actual explant used. Plant apical meristems make good explants for the cultivation of virus-free plants. Hence, this method is usually used to eliminate viruses in many species.

Embryo culture

Embryo culture in plant tissue culture involves the aseptic culture of plant embryos on a nutrient medium to obtain complete plantlets. This technique is crucial for rescuing embryos that might otherwise fail to develop, especially in cases of distant hybridization or when seeds are dormant or have underdeveloped embryos.

Organ culture

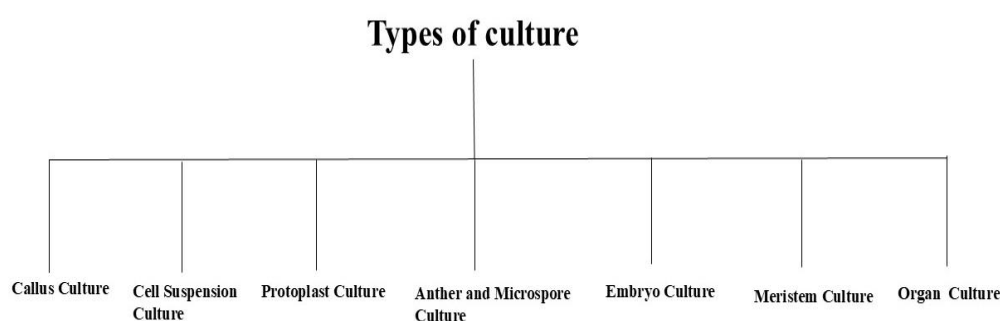
It is the cultivation of either whole organs or parts of organs in vitro. It is a development from [tissue culture](#) methods of research, as the use of the actual *in vitro* organ itself allows for more accurate modelling of the functions of an organ in various states and conditions.



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A key objective of organ culture is to maintain the architecture of the tissue and direct it towards normal development. In this technique, it is essential that the tissue is never disrupted or damaged. It thus requires careful handling. The media used for a growing organ culture are generally the same as those used for tissue culture. The techniques for organ culture can be classified into (i) those employing a solid medium and (ii) those employing liquid medium.

Organ culture technology has contributed to advances in embryology, inflammation, cancer, and stem cell biology research.



1.1.3 Aseptic techniques

- **Sterilization of Equipment and Media:**
 - **Dry Heat:** Used for sterilizing glassware and metal instruments using an autoclave or oven.
 - **Wet Heat:** Involves autoclaving (steam under pressure) to sterilize materials like culture media, glassware, and some instruments.
 - **Ultrafiltration:** A method for sterilizing heat-sensitive media components using membrane filters with small pore sizes.
 - **Chemical Sterilization:** Utilizing solutions like ethanol or bleach to disinfect surfaces and explants.
- **Sterilization of Explants:**



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- Surface sterilization of explants (small plant parts used for culture) is often achieved using chemical sterilants like bleach or ethanol, sometimes with a surfactant to aid penetration.
- **Maintaining a Sterile Environment:**
 - **Laminar Airflow Cabinets:** These create a sterile work area by providing a flow of HEPA-filtered air, minimizing airborne contamination.
 - **Transfer Rooms:** Dedicated spaces with controlled air quality and limited access help keep a sterile environment.
 - **Personal Hygiene:** Proper handwashing, wearing gloves and masks, and avoiding talking or coughing over cultures are important.
- **Aseptic Transfers:**
 - Inoculation (transferring explants to fresh media) should be done carefully, using sterilized instruments and flaming the necks of culture vessels to prevent contamination.
- **Monitoring and Troubleshooting:**
 - Regularly inspecting cultures for signs of contamination (e.g., fungal growth, bacterial cloudiness) is essential.

- If contamination occurs, it's important to identify the source and implement corrective measures.

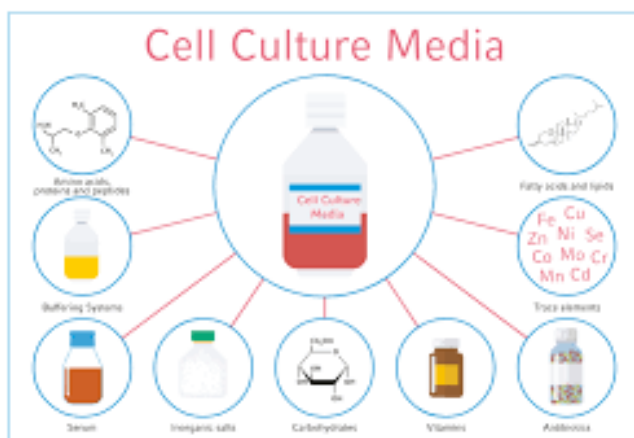


Fig: Key Components of Plant Tissue Culture Media

Summary

Plant Tissue Culture is a scientific technique used to grow and maintain plant cells, tissues, or organs under sterile and controlled conditions on a nutrient medium. It is based on the principle of **totipotency**, where a single plant cell has the potential to regenerate into a whole plant. The process involves the use of explants (small plant parts such as leaves, stems, or roots), which are cultured in nutrient media containing essential minerals, vitamins, plant hormones, and carbon sources. Plant tissue culture is widely applied in agriculture, horticulture, and biotechnology for **micropropagation, production of disease-free plants, conservation of rare and endangered species, genetic engineering, and secondary metabolite production**. It enables rapid multiplication of plants in large numbers, uniformity in plant traits, and year-round production irrespective of seasons, making it a vital tool for crop improvement and commercial plant production.



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Multiple Choice Questions

1. Plant tissue culture is based on the principle of:

- a) Totipotency
- b) Photosynthesis
- c) Transpiration
- d) Respiration

Answer: a) Totipotency

2. Which plant hormone is commonly used to induce root formation in tissue culture?

- a) Cytokinin
- b) Auxin
- c) Gibberellin
- d) Ethylene

Answer: b) Auxin

3. The nutrient medium used in plant tissue culture was first developed by:

- a) White
- b) Murashige and Skoog
- c) Haberlandt
- d) Skoog and Miller

Answer: b) Murashige and Skoog

4. The sterilizing agent commonly used for plant tissue culture is:

- a) Sodium hypochlorite
- b) Ethanol
- c) Mercuric chloride
- d) All of the above

Answer: d) All of the above

5. Callus is:



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- a) Differentiated tissue
- b) Mass of undifferentiated cells
- c) Specialized organ
- d) Stem cutting

Answer: b) Mass of undifferentiated cells

6. The process of growing a complete plant from a single cell in vitro is called:

- a) Cloning
- b) Organogenesis
- c) Somatic embryogenesis
- d) Micropropagation

Answer: c) Somatic embryogenesis

7. Cytokinins in tissue culture mainly promote:

- a) Root formation
- b) Shoot formation
- c) Leaf abscission
- d) Senescence

Answer: b) Shoot formation

8. Which of the following is an application of plant tissue culture?

- a) Micropropagation
- b) Germplasm conservation
- c) Production of secondary metabolites
- d) All of the above

Answer: d) All of the above

9. Protoplast culture is mainly used for:

- a) Somatic hybridization
- b) Root initiation
- c) Callus induction
- d) Organogenesis



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Answer: a) Somatic hybridization

10. The first scientist to attempt plant tissue culture was:

- a) Haberlandt
- b) Darwin
- c) Morgan
- d) Mendel

Answer: a) Haberlandt

Short answer type questions on Plant Tissue Culture:

1. What is plant tissue culture?
2. Define totipotency in plant cells.
3. Name two applications of plant tissue culture in agriculture.
4. What is callus?
5. Mention one difference between organ culture and cell culture.

Short-answer type questions on Plant Tissue Culture

1. What is plant tissue culture?
2. Who is known as the “Father of Plant Tissue Culture”?
3. Define the term **totipotency**.
4. What is an explant?
5. Name two applications of plant tissue culture.



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Unit 1.2.: Tissue Culture Media

- **Macronutrients:**

These are required in larger quantities for plant growth and include nitrogen, phosphorus, potassium, calcium, magnesium, and sulfur.

- **Micronutrients:**

These are needed in smaller amounts and include iron, boron, manganese, zinc, copper, molybdenum, and cobalt.

- **Vitamins:**

Vitamins like thiamine, pyridoxine, and nicotinic acid are essential for various metabolic processes.

- **Amino Acids and Other Nitrogen Supplements:**

These provide a source of nitrogen and other building blocks for protein synthesis.

- **Carbon Source:**

Typically, sucrose is used as a carbon source to provide energy for plant cells.

- **Growth Regulators:**

These include auxins and cytokinins, which stimulate cell division, differentiation, and organogenesis.

- **Solidifying Agent:**

Agar or agarose are commonly used to solidify the medium, creating a gel-like consistency that provides support for the plant tissues.

Common Examples of Media:

- **Murashige and Skoog (MS) Medium:**

One of the most widely used media for plant tissue culture, developed by Toshio Murashige and Folke Skoog in 1962.



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- **Gamborg's B5 Medium:**

Another popular medium, often used for cell suspension cultures and shoot regeneration.

Other Important Considerations:

- **Sterilization:**

Media must be sterilized, usually by autoclaving, to prevent contamination by microorganisms.

- **pH:**

The pH of the medium needs to be adjusted to the optimal level for plant growth.

- **Specific Requirements:**

The exact composition and concentration of each component may need to be adjusted depending on the specific plant species and the type of tissue culture being performed.

Liquid Media

Liquid media of plant tissue culture presents a solution of nutrients for the culture of plant cells, tissues, or organs under an in vitro controlled environment. It is one of the essential elements used for effective micropropagation and other plant tissue culture methods. Liquid medium delivers vital macronutrients, micronutrients, vitamins, carbohydrates, and plant growth regulators for the growth of plants.

Solid media

Solid media for plant tissue culture provides a solid or semi-solid support for plant tissues to grow in a controlled environment. Agar is the most commonly used gelling agent, providing structural support and stability to the medium. Other options include agarose, gellan gum, and phytigel.



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Semi solid media

Semi-solid media in plant tissue culture, also known as gelled or semi-gelled media, is a type of growth medium that has a soft, gel-like consistency. It's created by adding a gelling agent like agar or gellan gum to a liquid nutrient medium. This provides support for plant tissues while allowing for the exchange of nutrients and gases.

Specialized media

Generation of cryopreservation pre treatment

Cryopreservation pre-treatment in plant tissue culture aims to enhance the survival of plant cells, tissues, or organs during freezing and thawing. This involves techniques like cold hardening, desiccation, and the application of cryoprotectants, which are chemicals that protect cells from freezing damage. These pre-treatments are crucial for minimizing ice crystal formation and reducing cellular damage during the cryopreservation process.

2.1.1 Key pre-treatment strategies:

- **Cold Hardening:**

Exposing plant material to gradually decreasing temperatures to induce physiological changes that improve freezing tolerance. This can involve exposing plant tissues to low temperatures for a period of time, which can increase the concentration of cryoprotective substances within the cells.

- **Desiccation:**

Reducing the water content of the plant material to minimize ice crystal formation during freezing. This is often achieved by exposing the plant material to controlled humidity or using specific solutions that draw out water from the cells.



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- **Cryoprotectant Application:**

Utilizing chemicals that protect cells from damage during freezing and thawing. Common cryoprotectants include dimethyl sulfoxide (DMSO), glycerol, and sugars like sucrose. These substances help stabilize cell membranes and reduce the formation of harmful ice crystals.

Summary

Tissue culture media is the nutrient solution used for the growth and maintenance of plant or animal cells under controlled laboratory conditions. It provides essential nutrients, vitamins, hormones, and energy sources required for cell survival, division, and differentiation. A typical plant tissue culture medium, such as Murashige and Skoog (MS) medium, contains macronutrients (nitrogen, phosphorus, potassium, calcium, magnesium, sulfur), micronutrients (iron, manganese, zinc, boron, copper, molybdenum), vitamins (thiamine, nicotinic acid, pyridoxine), carbon source (usually sucrose), and a gelling agent (agar) for solid media. Growth regulators like auxins and cytokinins are added to regulate morphogenesis and organogenesis. The composition of the medium can be modified depending on the type of tissue, species, and the objective of culture (callus formation, shoot regeneration, root induction, or somatic embryogenesis). Thus, tissue culture media plays a vital role in successful in vitro propagation and biotechnological applications.

MCQs on Tissue Culture Media

1. Which of the following is the most commonly used basal medium in plant tissue culture?
 - a) MS medium
 - b) B5 medium
 - c) White's medium



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d) N6 medium

Answer: a) MS medium

2. In MS medium, the primary carbon source is:

a) Glucose

b) Sucrose

c) Maltose

d) Fructose

Answer: b) Sucrose

3. Which vitamin is most commonly used in tissue culture media?

a) Vitamin A

b) Vitamin B1 (Thiamine)

c) Vitamin D

d) Vitamin C

Answer: b) Vitamin B1 (Thiamine)

4. Cytokinins in tissue culture media promote:

a) Root formation

b) Shoot proliferation

c) Callus browning

d) Senescence

Answer: b) Shoot proliferation



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5. Which of the following is a synthetic auxin used in plant tissue culture media?

- a) IAA
- b) NAA
- c) IBA
- d) Zeatin

Answer: b) NAA

6. Agar is added to plant tissue culture media mainly to:

- a) Supply nutrients
- b) Provide hormones
- c) Solidify the medium
- d) Prevent contamination

Answer: c) Solidify the medium

7. The optimal pH of plant tissue culture media before autoclaving is:

- a) 4.0–4.5
- b) 5.6–5.8
- c) 6.5–7.0
- d) 7.5–8.0

Answer: b) 5.6–5.8

8. The role of activated charcoal in tissue culture medium is to:

- a) Supply vitamins
- b) Absorb inhibitory substances



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- c) Increase osmotic pressure
- d) Provide carbon source

Answer: b) Absorb inhibitory substances

9. The ratio of auxin to cytokinin in culture media determines:

- a) Nutrient uptake
- b) Organogenesis pathway
- c) Medium solidification
- d) pH stability

Answer: b) Organogenesis pathway

10. Which of the following is not a component of MS medium?

- a) Macronutrients
- b) Micronutrients
- c) Growth regulators
- d) Animal serum

Answer: d) Animal serum

short answer type questions on Tissue Culture Media:

1. What is tissue culture media?
2. Name the two main types of tissue culture media.
3. What are the basic components of a tissue culture medium?
4. Why are vitamins added to tissue culture media?
5. What is the role of sucrose in culture media?



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Unit 1.3: Growth Regulators

Plant growth regulators (PGRs), also known as plant hormones, are crucial in plant tissue culture for controlling and manipulating plant growth and development. They influence various processes like cell division, cell elongation, differentiation, and morphogenesis, thus playing a vital role in successful in vitro propagation.

- **Auxins:** Primarily responsible for cell elongation, root initiation, and callus formation.
- **Cytokinins:** Promote cell division, shoot formation, and help in overcoming apical dominance.
- **Gibberellins:** Stimulate cell elongation, flowering, and fruit development.



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- **Abscissic Acid (ABA):** Involved in dormancy, stress responses, and inhibiting shoot growth.
- **Ethylene:** Primarily associated with fruit ripening and senescence, but can also inhibit root formation in tissue culture.
- **Other PGRs:** Brassinosteroids and polyamines are also sometimes used, though less commonly.

1.3.1 Types of plant growth regulators

Auxin

Auxins are the important plant growth regulators, particularly in plant tissue culture. They significantly contribute to cell division, cell elongation, and differentiation and affect root, shoot development, and callus formation. In particular, auxins are vital for root induction, somatic embryogenesis and are capable of manipulating plant development in vitro.

Key Roles of Auxins in Plant Tissue Culture:

- **Root Induction:**

Auxins, like indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA), are widely used to stimulate root formation in plant tissue culture, particularly in the process of rooting cuttings.

- **Callus Formation:**

Auxins, in combination with other plant growth regulators, can induce callus formation, an undifferentiated mass of cells that can be further manipulated for various applications.

- **Somatic Embryogenesis:**

Auxins, such as 2,4-D, are often used to induce somatic embryogenesis, a process where embryos develop from somatic (non-reproductive) cells.

- **Organogenesis:**



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Auxins, along with other hormones like cytokinins, are involved in organogenesis, the process of forming organs like shoots and roots.

- **Cellular Processes:**

Auxins influence cell division, elongation, and differentiation, which are fundamental processes in plant tissue culture.

Examples of Auxins in Tissue Culture:

- **Indole-3-acetic acid (IAA):**

The most common natural auxin, IAA is involved in various developmental processes and is used in tissue culture for root induction and other applications.

- **Naphthalene acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D):**

These are synthetic auxins that are widely used in tissue culture for root induction, callus formation, and somatic embryogenesis.

Practical Applications:

- **Micropropagation:**

Auxins are essential for the successful micropropagation of plants, allowing for the rapid multiplication of plants from small tissue samples.

- **Genetic Transformation:**

Auxins can be used to manipulate plant development during genetic transformation experiments, influencing the regeneration of transformed cells.

- **Production of Secondary Metabolites:**

Tissue culture coupled with auxin treatment can be used to produce valuable secondary metabolites from plants.

Gibberellins



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Gibberellins (GAs) are a class of plant hormones that play a crucial role in plant growth and development, including their use in tissue culture. They are involved in stem elongation, seed germination, and flowering. In tissue culture, gibberellins, particularly GA₃, can be used to induce adventitious embryo formation and promote plantlet development, but their effect can vary, and they may inhibit organ development in some cases.

1.3.2 Role of Gibberellins in Plant Tissue Culture:

- **Inducing Adventitious Embryos:**

Gibberellins, especially GA₃, are often used to stimulate the formation of adventitious embryos in tissue culture, which can then develop into plantlets.

- **Promoting Plantlet Formation:**

They can also promote the development of plantlets from these embryos, leading to successful plant propagation.

- **Varying Effects:**

The effectiveness of gibberellins in tissue culture can vary depending on the plant species, tissue type, and the specific culture conditions.

- **Potential Inhibition:**

In some cases, gibberellins can inhibit organ development (root and shoot formation) and somatic embryogenesis, making it crucial to optimize their concentration and application.

- **Stem Elongation:**

Gibberellins promote stem elongation, which can be beneficial in tissue culture for achieving desired plant architecture.

- **Seed Germination:**

They are also involved in seed germination, which can be important for establishing a new culture or regenerating plants from seeds.



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- **Optimizing Concentration:**

It's crucial to optimize the concentration of gibberellins in the culture medium to achieve the desired outcome, as different plant species and tissues respond differently to these hormones.

- **Combination with other PGRs:**

Gibberellins are often used in combination with other plant growth regulators (PGRs), such as auxins and cytokinins, to achieve specific developmental outcomes in tissue culture.

- **Auxin-Cytokinin Balance:**

The balance between auxins and cytokinins is crucial in tissue culture, with a high auxin to cytokinin ratio favoring root formation and a high cytokinin to auxin ratio favoring shoot formation.

Cytokinin

In plant tissue culture, cytokinins are essential plant growth regulators that promote cell division and differentiation, particularly for shoot development. They are often used in conjunction with auxins to control the growth and development of plant cells and tissues in a controlled, sterile environment. Cytokinins can induce shoot formation from undifferentiated callus and promote the growth of axillary buds.

Cytokinins in Plant Tissue Culture:

- **Promote Cell Division and Shoot Development:**

Cytokinins are known for their ability to stimulate cell division, which is crucial for initiating and propagating new shoots in tissue culture.

- **Regulate Shoot Formation:**

In combination with auxins, cytokinins can be used to control the type and amount of shoot formation, allowing for the efficient propagation of plants with desired traits.

- **Induce Axillary Bud Growth:**



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Cytokinins play a role in breaking apical dominance, encouraging the growth of lateral or axillary buds, which can be beneficial in tissue culture for producing multiple shoots.

- **Delay Leaf Senescence:**

Cytokinins can help delay the aging of leaves in tissue culture, keeping them healthy and viable for longer periods.

- **Examples of Cytokinins Used in Tissue Culture:**

Common cytokinins used in plant tissue culture include benzyladenine (BA), kinetin, and zeatin, among others.

- **Balancing with Auxins:**

The ratio of cytokinins to auxins in the culture medium is crucial for determining the type of growth that occurs. High cytokinin levels generally favor shoot formation, while high auxin levels favor root formation.

In essence, cytokinins are vital tools in plant tissue culture, enabling the efficient propagation of plants by stimulating cell division, promoting shoot development, and influencing various other aspects of plant growth and development.

Ethylene

Ethylene, a gaseous plant hormone, can both inhibit and promote growth in plant tissue culture, depending on its concentration and the plant species. While not a standard growth regulator in many tissue culture protocols, ethylene can significantly impact plant development, particularly in root and shoot formation, and interact with other hormones like auxin. Its effects are complex, and high concentrations can be detrimental to explant responses, while low concentrations might enhance responses to other hormones.

Here's a more detailed explanation:

Ethylene's Dual Role in Tissue Culture:



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- **Inhibition:**

At higher concentrations, ethylene can inhibit growth and development in tissue culture, potentially hindering the establishment and proliferation of plantlets.

- **Promotion:**

At lower concentrations, ethylene can promote root and shoot development, influencing factors like leaf elongation and overall plant differentiation.

- **Interaction with other hormones:**

Ethylene can interact with other plant growth regulators, particularly auxin, to influence various developmental processes in tissue culture.

Ethylene's Impact on Plant Development:

- **Root and Shoot Development:**

Ethylene can promote or inhibit root and shoot formation, depending on its concentration and the specific plant species.

- **Senescence and Abscission:**

Ethylene is known to promote senescence (aging) and abscission (leaf or fruit drop), which can be relevant in tissue culture depending on the objectives.

- **Flowering:**

In some cases, ethylene can induce flowering, which might be desirable in specific tissue culture applications.

Managing Ethylene in Tissue Culture:

- **Ventilation:**

Because ethylene is a gas, proper ventilation in culture vessels is crucial to prevent its accumulation, which can negatively impact explant responses.



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- **Ethylene Scavengers:**

Chemicals like Ethephon (Ethrel) or Purafil are sometimes used to absorb or neutralize ethylene in the culture environment.

- **Silver Nitrate:**

Silver nitrate is a common additive in tissue culture media that can inhibit ethylene action and improve plant regeneration in some cases.

Absciscic acid

Absciscic acid (ABA) is a plant growth regulator that plays a significant role in plant tissue culture, particularly in promoting somatic embryogenesis and enhancing the quality of somatic embryos. It can also be used to induce a quiescent state in somatic embryos, improve in vitro conservation, and enhance the adaptive response of plant cells and tissues to various stresses.

Here's a more detailed explanation:

Role of Absciscic Acid in Tissue Culture:

- **Somatic Embryogenesis:**

ABA is crucial in the induction and maturation of somatic embryos, which are embryos derived from somatic (non-reproductive) cells. It helps in the development of these embryos and influences their quality.

- **Stress Tolerance:**

ABA is often referred to as the "stress hormone" because it is involved in plant responses to various environmental stresses like drought, salinity, and temperature extremes. In tissue culture, ABA can be used to enhance the tolerance of plant cells and tissues to these stresses, improving their survival and growth under unfavorable conditions.

- **Seed Dormancy and Germination:**

ABA plays a key role in maintaining seed dormancy and inhibiting premature germination. In tissue culture, this can be useful for regulating the development and germination of somatic embryos.



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- **In Vitro Conservation:**

ABA can be used to improve the in vitro conservation of plant tissues by inducing a quiescent state in the cultured cells or tissues. This can help in long-term storage and preservation of valuable plant germplasm.

- **Modulating Auxin Effects:**

ABA can modulate the effects of other plant growth regulators like auxins, which are involved in cell growth and differentiation. In tissue culture, this interaction can be utilized to optimize the developmental pathways and improve the efficiency of certain processes like shoot regeneration.

Peptide hormone

Peptide hormones play a crucial role in plant tissue culture, influencing various developmental processes. They act as signaling molecules, coordinating cell-to-cell communication and regulating growth and development. In tissue culture, these hormones, along with classical plant hormones like auxins and cytokinins, are essential for successful plant regeneration and organogenesis.

Key Roles of Peptide Hormones in Plant Tissue Culture:

- **Shoot and Root Development:**

Peptide hormones, like CLE and CEP peptides, are involved in shoot and root development, influencing meristem maintenance, lateral root formation, and root hair development.

- **Organogenesis:**

Cytokinin, a type of plant hormone, are particularly important for shoot organogenesis in tissue culture.

- **Plant Regeneration:**

Peptide hormones, in conjunction with other plant hormones, are crucial for the regeneration of plants from explants in tissue culture.



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- **Cellular Processes:**

Peptide hormones regulate various cellular processes, including cell division, differentiation, and cell expansion.

Signal transduction pathways

Signal transduction pathways in plant tissue culture involve a series of molecular events that allow cells to perceive and respond to both internal and external signals, ultimately influencing cellular processes like growth, development, and differentiation. These pathways are crucial for somatic organogenesis, plant regeneration, and other aspects of plant tissue culture.

Signal Reception:

- **Receptors:**

Plants have receptors, often proteins, located on the cell membrane or within the cell, that bind to specific signaling molecules (ligands). These ligands can be plant hormones, environmental cues (like light or temperature), or even molecules released by other cells.

- **Specificity:**

Receptors are highly specific, meaning they only bind to certain signaling molecules, ensuring the correct signal is received.

- **Examples:**

Receptors can include receptor kinases (like BRI1 for brassinosteroids) or G protein-coupled receptors.

2. Signal Transduction:

- **Second Messengers:**

Once a receptor is activated, it triggers the production or release of intracellular signalling molecules called second messengers.

- **Amplification:**



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Second messengers amplify the initial signal, allowing for a stronger and more widespread cellular response.

- **Examples:**

Common second messengers include calcium ions (Ca^{2+}), cyclic nucleotides (like cyclic GMP), and reactive oxygen species (ROS).

3. Cellular Response:

- **Protein Kinases:**

Activated second messengers often activate protein kinases, which are enzymes that modify other proteins by adding phosphate groups (phosphorylation).

- **Transcription Factors:**

Phosphorylation can activate or inactivate transcription factors, which are proteins that bind to DNA and regulate gene expression.

- **Cellular Changes:**

These changes in gene expression can lead to various cellular responses, such as:

- **Growth and Development:** Cell division, differentiation, and morphogenesis.
- **Stress Response:** Production of stress proteins, changes in metabolism, etc.
- **Hormone Response:** Regulating plant hormone levels and signaling pathways.

Specific Examples in Tissue Culture:

- **Cytokinin Signaling:**

Cytokinins play a critical role in shoot organogenesis and cell division in tissue culture. The cytokinin receptor histidine kinase (AHK) perceives the signal, leading to the activation of response regulators



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(ARRs) that ultimately regulate gene expression related to shoot development.

- **Auxin Signaling:**

Auxins are also crucial for plant regeneration, particularly for root formation and the development of vascular tissue. Auxin signaling pathways involve receptors like TIR1 and AUX/IAA proteins, which regulate gene expression related to cell growth and differentiation.

- **Brassinosteroid Signaling:**

Brassinosteroids (BRs) are important for cell elongation and differentiation. The receptor BRI1 perceives BRs, and this signal is transduced through a pathway involving BSU1, BIN2, and BES1, ultimately affecting gene expression related to cell growth.

- **Environmental Signals:**

Factors like light and temperature can also affect plant tissue culture responses through their respective signal transduction pathways. For example, phytochrome, a light receptor, plays a role in regulating gene expression related to chloroplast development and other aspects of plant development.



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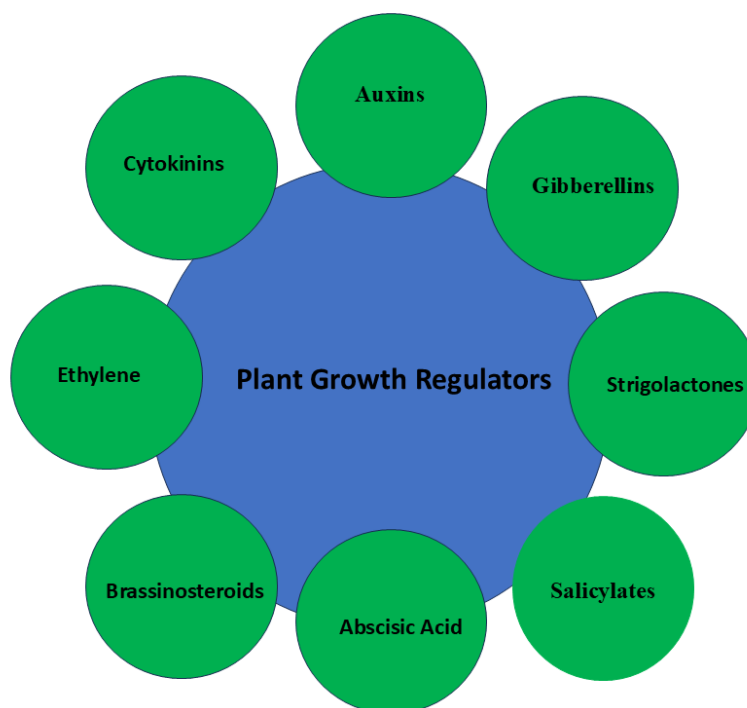


Fig: Different types of plant hormones

Application of growth regulators in agriculture and horticulture

Summary

Introduction to Plant Tissue Culture – Summary (Paragraph):

Plant tissue culture is a technique used to grow plant cells, tissues, or organs under sterile conditions on a nutrient culture medium. It is based on the principle of *totipotency*, which means that every plant cell has the potential to regenerate into a whole plant. This method is widely used in research, agriculture, and horticulture for the rapid multiplication of plants, conservation of rare and endangered species, production of disease-free plants, and

conditions. It plays a vital role in plant biotechnology and modern crop improvement programs.

MCQs:



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1. What is the term for the process of growing plant cells or tissues in

a controlled environment?

- a) Micropropagation
- b) Tissue Culture
- c) Cloning
- d) Germination

2. Which technique is essential for ensuring that a tissue culture remains free from microbial contamination?

Aseptic technique

- b) In-vitro technique
- c) Genetic modification
- d) Somatic hybridization

3. What is the main component of a basic tissue culture media?

- a) Agar
- b) Water
- c) Plant hormones
- d) All of the above

4. Which of the following is a plant growth regulator?

- a) Cytokinin
- b) Glucose
- c) Phosphorus
- d) Nitrogen

5. What is the role of auxins in plant tissue culture?

- a) Root initiation



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- b) Leaf growth
- c) Flower production
- d) Fruit ripening

6. Which media is commonly used for growing plant cells in culture?

- a) Murashige and Skoog medium
- b) Nitrate medium
- c) Selective medium
- d) All of the above

7. What type of plant growth regulator is used to promote cell division?

- a) Auxin
- b) Cytokinin
- c) Gibberellin
- d) Ethylene

8. What is the general purpose of tissue culture in agriculture?

- a) Increasing yield
- b) Propagating plants
- c) Increasing biodiversity
- d) All of the above

9. Which of the following is NOT a part of tissue culture medium?

- a) Plant cells
- b) Vitamins
- c) Minerals
- d) Hormones



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10. What type of media composition is essential for plant tissue culture?

- a) Balanced nutrients
- b) Excess of minerals
- c) Low oxygen levels
- d) High sugar levels

Short Questions:

1. Define plant tissue culture.
2. List the different types of tissue cultures.
3. What are aseptic techniques in plant tissue culture?
4. Explain the composition of tissue culture media.
5. Mention two types of growth regulators used in tissue culture.
6. What is the role of cytokinin in tissue culture?
7. How does the Murashige and Skoog medium help in plant tissue culture?
8. Why is sterilization important in plant tissue culture?
9. What is organogenesis in tissue culture?
10. Describe the importance of growth regulators in agricultural applications.

Long Questions:

1. Discuss the types of plant tissue culture techniques.
2. Explain the composition of tissue culture media and their significance.
3. Describe the role of growth regulators in plant tissue culture and their applications.



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4. How do aseptic techniques contribute to the success of plant tissue culture?
5. What are the recent advancements in tissue culture media?
6. How do plant growth regulators like auxins and cytokinins interact in tissue culture?
7. Discuss the applications of tissue culture in agriculture and horticulture.
8. Explain how the growth of plant tissues is regulated through media and hormones.
9. Discuss the various types of plant growth regulators used in tissue culture.
10. Explain the importance of tissue culture in the development of disease-resistant crops.

Module 2

Callus culture, Cell suspension

Objective

To explore the techniques and applications of callus culture, cell suspension culture, organogenesis, somatic embryogenesis, and micropropagation.

Unit 2.1: Callus Culture

2.1.1 Definition and Overview of Callus Culture

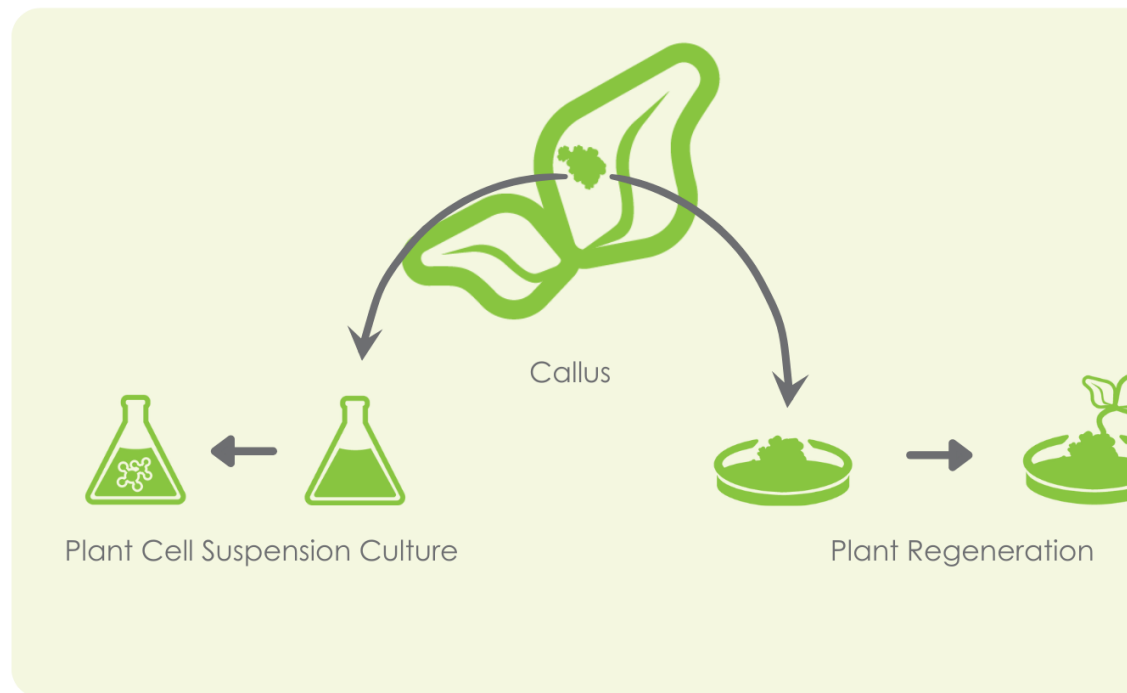


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Callus culture is a plant tissue culture method in which a collection of undifferentiated plant cells (callus) is cultured on a nutrient medium in the laboratory. Wounding plant material or employing certain plant hormones is commonly used to induce this process. Callus cultures are useful for cellular studies, inducing genetic variation, and even for regenerating whole plants. Plant species within each of the major land plant groups are able to form callus in tissue culture. A cell culture of callus is typically maintained on gel medium. The callus induction medium is made up of agar and a combination of macronutrients and micronutrients for the specific cell type. There are a number of basal salt mixtures that are employed in plant tissue culture, but most prominently altered Murashige and Skoog medium, White's medium and woody plant medium. Vitamins, like Gamborg B5 vitamins are also supplemented to promote growth. For plant cells, the enrichment with nitrogen, phosphorus, and potassium is particularly significant. Plant callus is typically obtained from somatic tissues. The tissues from which callus induction is initiated vary according to plant species and available tissues for explant culture. The cells that form callus and somatic embryos tend to rapidly divide or are partially undifferentiated like meristematic tissue. In alfalfa (*Medicago truncatula*), however, the callus and somatic embryos arise from dedifferentiating mesophyll cells. Plant hormones induce callus growth. Once the callus is established, the hormone concentration in the medium can be changed to direct further development towards root organogenesis, shoot formation, or somatic embryogenesis. The callus tissue subsequently develops further cell growth and differentiation into the respective organ primordia. The matured organs can then be employed in the regeneration of new mature plants.



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Detailed Types:

- **Friable Callus:**
 - Characterized by loosely aggregated cells.
 - Easily disperses into a cell suspension culture.
 - Can be white, cream, or yellowish in color.
- **Compact Callus:**
 - Consists of densely packed cells.
 - Preferred for regeneration studies due to its organized structure.
 - Typically green and sturdy.
- **Callus for Plant Regeneration:**
 - **Shoot callus:** Forms shoots.
 - **Root callus:** Forms roots.
 - **Embryogenic callus:** Forms embryos, which can develop into complete plants.



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- **Callus for Other Purposes:**
 - Used for metabolite production or other specialized applications.
- **Other Descriptive Terms:**
 - **Nodular Callus:** Contains organized structures resembling meristems, indicating a tendency towards differentiation.
 - **Wound-induced Callus:** Forms in response to trauma or injury to the plant.
 - **Hormone-induced Callus:** Formed by the application of specific plant hormones (auxins and cytokinins).

Factors Affecting Callus Growth and Differentiation:

- **Plant Growth Regulators (PGRs):**
 - **Auxins and Cytokinins:** These hormones are fundamental in regulating cell division and differentiation. The ratio of auxins to cytokinins in the medium greatly impacts callus development. High auxin to cytokinin ratios favor root formation, while high cytokinin to auxin ratios promote shoot formation. A balanced ratio can maintain callus in an undifferentiated state.
 - **Specific PGRs:** Different PGRs like NAA, 2,4-D, BAP, Kinetin, and TDZ can be used in various combinations to optimize callus induction and differentiation.
- **Nutrient Medium Composition:**
 - **Macronutrients and Micronutrients:** The specific types and concentrations of nutrients in the medium are critical for callus growth.



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- **Carbon Source:** Sugars like sucrose provide energy for cell growth and are a vital component of the medium.
- **Explant Source:**
 - **Plant Species:** Different plant species and even different genotypes within the same species can exhibit varying responses to callus induction and differentiation.
 - **Explant Type:** The type of plant tissue used as the explant (e.g., leaf, stem, root) can affect callus formation and its potential for differentiation.
- **Environmental Conditions:**
 - **Temperature:** Temperature plays a role in callus growth and development. Subculturing is often recommended every 4-6 weeks when incubated at 25°C.
 - **Light:** Light intensity and photoperiod can affect callus growth and differentiation.
- **Other Factors:**
 - **Gas Phase:** The gas exchange between the culture and the surrounding environment can also influence callus growth.
 - **Subculturing:** Regular subculturing on fresh media is necessary to prevent nutrient depletion and waste product buildup.

Application of Callus Culture in Plant Regeneration

- **Plant Regeneration and Micropropagation:** Callus culture is used for clonal propagation of elite genotypes, especially those difficult to propagate by conventional methods. Somatic embryogenesis (formation of embryos from somatic cells) and



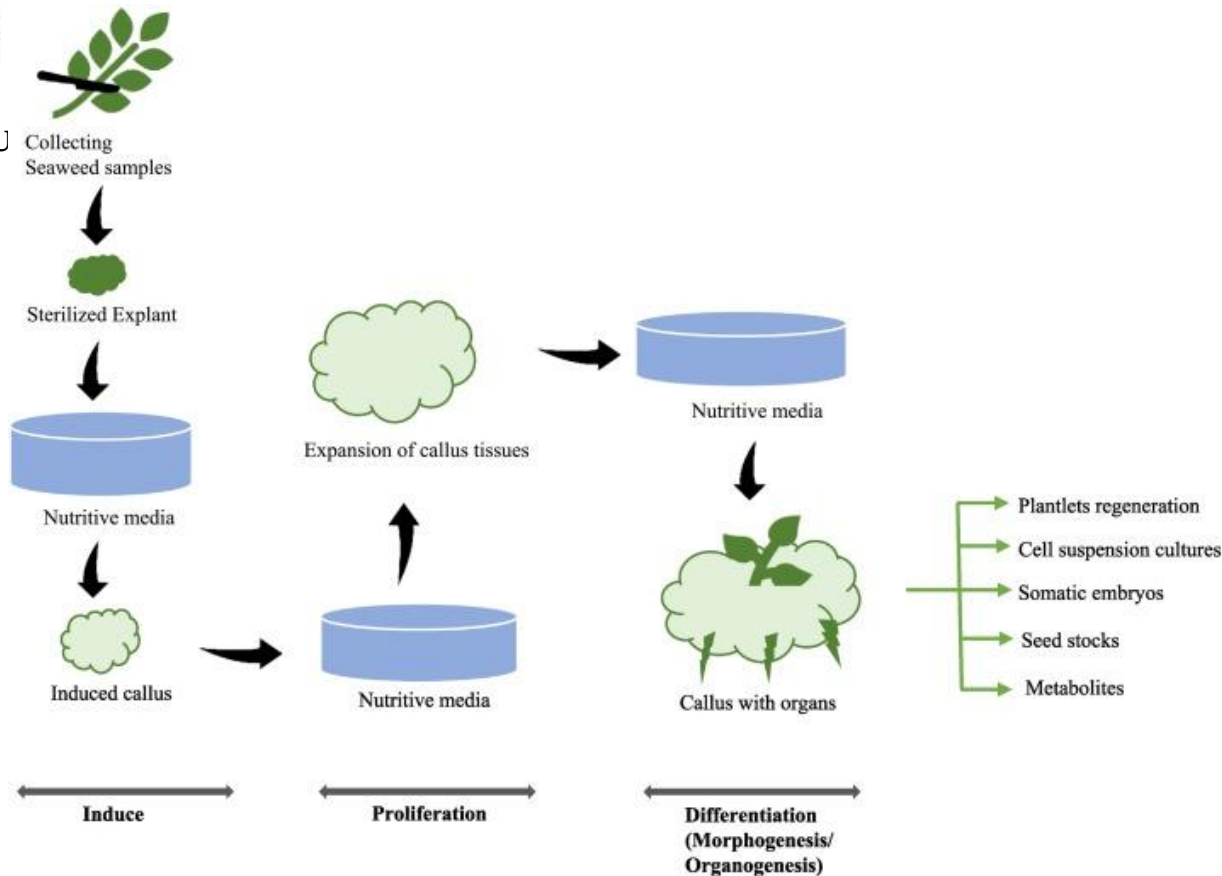
PLANT TISSUE CULTURE

organogenesis (formation of organs from callus) are key processes in plant regeneration.

- **Secondary Metabolite Production:** Callus cultures can be used to produce valuable secondary metabolites, such as pharmaceuticals, flavors, and fragrances. This offers a sustainable alternative to extraction from wild plants.
- **Genetic Transformation:** Callus is a target tissue for genetic engineering techniques like *Agrobacterium*-mediated transformation or gene gun bombardment. The transformed callus can then be regenerated into genetically modified plants.
- **Germplasm Conservation:** Callus cultures can be cryopreserved (frozen) for long-term storage, providing a valuable tool for conserving endangered plant species and valuable germplasm.
- **Disease Elimination:** Callus culture can be used to eliminate viruses and other pathogens from infected plants.



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Summary

Callus culture is a plant tissue culture technique where undifferentiated, unorganized masses of cells (called callus) are induced to grow from explants such as leaf, stem, or root tissues under sterile conditions. The process involves placing explants on a nutrient medium containing an appropriate balance of plant growth regulators, mainly auxins and cytokinins. Callus formation occurs as a result of dedifferentiation of plant cells, which regain the ability to divide. Callus cultures are widely used in plant biotechnology for micropropagation, somaclonal variation, genetic transformation, and the production of secondary metabolites. They also serve as a precursor step for organogenesis and somatic embryogenesis, making them an essential tool in the development of transgenic plants and in large-scale clonal propagation.



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MCQs on Callus Culture

1. Callus culture refers to:

- a) Culture of roots in vitro**
- b) Culture of unorganized mass of cells**
- c) Culture of shoots in vitro**
- d) Culture of flowers in vitro**

Answer: b) Culture of unorganized mass of cells

2. Callus is usually induced from plant tissues by using:

- a) Cytokinins alone**
- b) Auxins alone**
- c) A combination of auxins and cytokinins**
- d) Gibberellins**

Answer: c) A combination of auxins and cytokinins

3. Which explant is most commonly used for callus induction?

- a) Leaf disc**
- b) Root tip**
- c) Stem nodes**
- d) Flower petals**

Answer: a) Leaf disc

4. Callus tissue is characterized as:



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- a) Organized, differentiated tissue
- b) Undifferentiated, unorganized tissue
- c) Specialized reproductive tissue
- d) Fully differentiated tissue

Answer: b) Undifferentiated, unorganized tissue

5. For rapid proliferation of callus, the medium should contain:

- a) Only cytokinins
- b) High auxin concentration
- c) Balanced auxin and cytokinin ratio
- d) Only gibberellins

Answer: c) Balanced auxin and cytokinin ratio

6. The first scientist to demonstrate callus culture was:

- a) Haberlandt
- b) White
- c) Skoog and Miller
- d) Murashige and Skoog

Answer: b) White

7. Callus can be induced from which type of plant tissue?

- a) Meristematic tissue only
- b) Parenchymatous tissue
- c) Differentiated tissue



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d) Any living plant tissue with totipotency

Answer: d) Any living plant tissue with totipotency

8. Friable callus is important because it can be used for:

a) Somatic embryogenesis

b) Suspension culture

c) Organogenesis

d) Both a and b

Answer: d) Both a and b

9. Which of the following is NOT an application of callus culture?

a) Production of secondary metabolites

b) Clonal propagation

c) Crop improvement via somaclonal variation

d) Direct seed germination

Answer: d) Direct seed germination

10. The medium most widely used for callus culture is:

a) White's medium

b) Murashige and Skoog (MS) medium

c) Nitsch medium

d) Knop's medium

Answer: b) Murashige and Skoog (MS) medium



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short answer type questions on Callus Culture:

- 1. What is callus culture in plant tissue culture?**
- 2. How is callus induced from plant explants?**
- 3. Name two common plant growth regulators used for callus induction.**
- 4. What is the role of auxins in callus culture?**
- 5. Mention one advantage of callus culture.**

Unit 2.2: Organogenesis and Somatic Embryogenesis

In plant tissue culture, organogenesis and somatic embryogenesis are two distinct pathways for plant regeneration. Organogenesis involves the formation of plant organs (shoots, roots) from an explant or callus, while somatic embryogenesis results in the development of embryo-like structures from somatic cells. Both processes are crucial for micropropagation and genetic modification of plants.

Organogenesis:

- Definition:**



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The development of plant organs (shoots, roots, etc.) from a mass of undifferentiated cells called callus or directly from an explant.

- **Process:**

Typically involves dedifferentiation of explant cells into callus, followed by redifferentiation into specific organs, often guided by plant growth regulators.

- **Types:**

Can be direct (without callus formation) or indirect (through callus formation).

- **Applications:**

Used for plant propagation, genetic transformation, and in vitro conservation.

Somatic Embryogenesis:

- **Definition:**

The formation of embryo-like structures (somatic embryos) from somatic cells, which can develop into whole plants.

- **Process:**

Mimics natural embryo development, with stages like initiation, development, maturation, and germination.

- **Types:**

Can be direct (embryos form directly from cells) or indirect (embryos form from callus).

- **Applications:**

Used for producing artificial seeds, plant propagation, and in various research areas like genetic analysis and metabolite production.

Unit 06: Micropropagation



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Micropropagation, also known as clonal propagation or in vitro propagation, is a technique used in plant tissue culture to rapidly multiply plants from small pieces of tissue (explants) under sterile conditions. This method allows for the production of a large number of genetically identical plants (clones) in a short amount of time and space, making it a valuable tool for various applications in agriculture, horticulture, and plant conservation.

Key aspects of micropropagation:

- **Aseptic conditions:**

Micropropagation is performed under sterile conditions to prevent contamination by microorganisms like bacteria and fungi.

- **Explants:**

Small pieces of plant tissue, such as shoot tips, buds, or nodes, are used as explants to initiate the process.

- **Growth media:**

The explants are grown on a nutrient-rich medium containing essential minerals, vitamins, and hormones to support growth and development.

- **Stages of micropropagation:**

The process typically involves several stages:

- **Stage 0: Selection of mother plant:** Choosing a healthy, disease-free plant as the source for explants.
- **Stage I: Establishment:** Sterilizing the explants and placing them on a nutrient medium to initiate growth.
- **Stage II: Multiplication:** Promoting shoot development and multiplication through the use of growth hormones.
- **Stage III: Rooting:** Inducing root formation on the multiplied shoots.



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- **Stage IV: Acclimatization:** Gradually adapting the plantlets to the natural environment by transferring them from sterile conditions to soil.

- **Advantages:**

Micropropagation offers several advantages over traditional methods, including:

- **Rapid multiplication:** Producing a large number of plants in a short period.
- **Genetic uniformity:** Ensuring that all plants are genetically identical (clones).
- **Disease-free plants:** Reducing the risk of transmitting diseases or pests.
- **Year-round production:** Enabling plant propagation regardless of the season.
- **Conservation of rare and endangered species:** Facilitating the propagation of plants that are difficult to propagate through traditional methods.

- **Applications:**

Micropropagation is widely used in:

- **Commercial plant production:** Producing plants for agriculture, horticulture, and floriculture.
- **Germplasm conservation:** Preserving valuable plant genetic resources.
- **Research and development:** Studying plant development, gene expression, and other biological processes.

- **Limitations:**



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Micropropagation can be expensive and labor-intensive, requiring specialized facilities and trained personnel.

Summary

Organogenesis and somatic embryogenesis are two key morphogenetic pathways in plant tissue culture used for plant regeneration.

- **Organogenesis** is the process by which shoots and roots are formed from explants or callus under controlled in vitro conditions. It may occur **directly** (from explant tissues without an intermediate callus phase) or **indirectly** (through callus formation first). Plant growth regulators play a crucial role: auxins generally promote root formation, while cytokinins induce shoot formation. This technique is widely used for micropropagation and clonal multiplication of plants.
- **Somatic Embryogenesis** is the process of developing embryos from somatic (non-reproductive) cells instead of zygotes. These somatic embryos resemble zygotic embryos in morphology and developmental stages (globular, heart, torpedo, cotyledonary). It can also be **direct** (embryos arise directly from explant cells) or **indirect** (via callus). Somatic embryogenesis is important for large-scale propagation, synthetic seed production, and genetic transformation studies.

MCQs on Organogenesis

Organogenesis in plant tissue culture refers to:

- a) Formation of somatic embryos from somatic cells



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- b) Formation of organs like shoots and roots from cultured cells/tissues
- c) Formation of callus from explants
- d) Fusion of protoplasts

Answer: b) Formation of organs like shoots and roots from cultured cells/tissues

Which growth regulator promotes shoot organogenesis?

- a) Auxin in high concentration
- b) Cytokinin in high concentration
- c) Gibberellins
- d) Absciscic acid

Answer: b) Cytokinin in high concentration

Root organogenesis is generally induced by:

- a) High cytokinin and low auxin
- b) High auxin and low cytokinin
- c) Equal auxin and cytokinin
- d) No hormones required

Answer: b) High auxin and low cytokinin

The callus that gives rise to organs is called:

- a) Non-morphogenic callus
- b) Morphogenic callus
- c) Embryogenic callus
- d) Dedifferentiated callus



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Answer: b) Morphogenic callus

Direct organogenesis occurs when:

- a) Organs develop directly from explant without callus phase
- b) Organs develop after callus formation
- c) Somatic embryos form without hormones
- d) Fusion of gametes takes place

Answer: a) Organs develop directly from explant without callus phase

MCQs on Somatic Embryogenesis

Somatic embryogenesis is defined as:

- a) Formation of gametes from somatic cells
- b) Development of embryos from somatic or non-reproductive cells
- c) Embryo formation only from zygote
- d) Root formation from callus

Answer: b) Development of embryos from somatic or non-reproductive cells

Somatic embryos are:

- a) Morphologically identical to zygotic embryos
- b) Genetically identical to parent plant
- c) Can develop into a whole plant
- d) All of the above

Answer: d) All of the above



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The plant growth regulator most important for induction of somatic embryogenesis is:

- a) Auxin (2,4-D)
- b) Cytokinin
- c) Gibberellin
- d) Absciscic acid

Answer: a) Auxin (2,4-D)

Somatic embryos can arise:

- a) Directly from explant tissues
- b) Indirectly via callus phase
- c) Both (a) and (b)
- d) Only through sexual reproduction

Answer: c) Both (a) and (b)

Which of the following is a major application of somatic embryogenesis?

- a) Synthetic seed production
- b) Production of polyploids
- c) Somaclonal variation
- d) Protoplast fusion

Answer: a) Synthetic seed production



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Short answer type questions on **Organogenesis and Somatic Embryogenesis**

- What is organogenesis in plant tissue culture?
- Differentiate between direct and indirect organogenesis.
- Name two plant growth regulators that promote shoot organogenesis.
- What is somatic embryogenesis?
- Differentiate between direct and indirect somatic embryogenesis.

Unit 2.3 : Shoot-tip and Meristem Culture

Introduction to Shoot-tip and Meristem Culture

Shoot-tip and meristem culture are plant tissue culture methods aimed at using the actively dividing shoot tip cells of the shoot apex to produce new plants, frequently for virus eradication or quick propagation. Shoot-tip culture is done by culturing a portion of the shoot tip containing the meristem, whereas meristem culture utilizes only the dome-shaped meristematic tissue itself. These methods prove beneficial in delivering disease-free plants and clonal propagation.

Shoot-tip Culture:

- **Definition:**

Involves culturing the terminal portion of a shoot, typically 0.1-1.0 mm in length, which includes the meristem (0.05-0.1 mm) along with developing leaf primordia and adjacent stem tissue.

- **Principle:**



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Many viruses are unable to infect the apical meristem, making it possible to regenerate virus-free plants from small shoot tips.

- **Applications:**
 - **Virus elimination:** Especially effective for viruses that don't readily move between cells.
 - **Micropropagation:** Rapidly produces multiple copies of a plant.
 - **Preserving genetic resources:** Provides a way to store plant material while minimizing the risk of disease.

Meristem Culture:

- **Definition:**

Involves culturing the dome-shaped meristematic tissue, which is usually less than 0.1 mm in length, and only one or two pairs of the youngest leaf primordia.

- **Principle:**

Similar to shoot tip culture in that it aims to regenerate plants from tissues with high division rates, potentially free from pathogens.

- **Applications:**
 - **Virus elimination:** Highly effective for producing disease-free plants.
 - **Micropropagation:** Provides a method for rapid, clonal multiplication of plants.
 - **Other pathogen elimination:** Can also eliminate other pathogens like bacteria and fungi.

Explant Sources for Shoot-tip and Meristem Culture



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In plant tissue culture, explants like shoot tips and meristems are crucial for regeneration, particularly for virus elimination and rapid multiplication. Shoot tips, including the apical and axillary meristems, are frequently used for micropropagation due to their high regenerative potential. Meristem tips, even smaller than shoot tips, are favored for obtaining virus-free plants.

Explant Sources:

- **Shoot Tips:**

These are actively growing tips of shoots, including both apical (terminal) and axillary (lateral) buds. They are chosen for their high regenerative capacity and suitability for micropropagation.

- **Meristem Tips:**

These are even smaller than shoot tips, consisting of the meristematic dome and a few leaf primordia. They are particularly useful for producing virus-free plants due to the low or absent virus presence in actively dividing meristematic cells.

- **Nodal Segments:**

These are sections of the stem containing nodes (where leaves and buds arise). They can be used for micropropagation and can be a good source of axillary buds.

- **Other explants:**

While less common for shoot tip and meristem culture, other plant parts like leaves, roots, and even seeds can be used for different types of tissue culture applications.

Application of Shoot-tip and Meristem Culture

Shoot tip and meristem culture are valuable techniques in plant tissue culture with applications in producing virus-free plants, micropropagation, and plant conservation. Meristem culture involves



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using the apical or axillary meristems (0.1-0.3mm) for in vitro propagation, while shoot tip culture utilizes a larger portion of the shoot tip, including the meristem and developing leaves.

Key Applications:

- **Virus Elimination:**

Meristem culture is particularly effective in eliminating viruses from infected plants because many viruses cannot penetrate the meristematic tissue. This is crucial for producing disease-free planting material for various crops.

- **Micropropagation:**

Both shoot tip and meristem cultures are used for rapid clonal multiplication of plants, especially those that are difficult to propagate through conventional methods or are vegetatively propagated.

- **Plant Conservation:**

Meristem culture plays a vital role in conserving rare or endangered plant species and in the long-term storage of germplasm, especially for plants with short-lived seeds or those that are highly heterozygous.

- **Genetic Transformation:**

Meristem or shoot tip cultures can be used as target tissue for introducing new genes into plants, enabling the development of plants with improved traits like disease resistance or enhanced yield.

Summary

Callus culture and cell suspension culture are two important techniques in plant tissue culture used for research, genetic engineering, and large-scale production of plant compounds. In **callus culture**, plant tissues (usually from leaves, stems, or roots) are placed on a nutrient medium with specific hormones, leading to the formation of an unorganized mass of undifferentiated cells called a callus. This callus can be maintained or induced to regenerate into whole plants under suitable



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conditions. Cell suspension culture is derived from callus tissue, where small pieces of callus are transferred to a liquid medium and kept under constant agitation to produce a uniform suspension of free cells or small cell clusters. This method is highly useful for studying cell biology, producing secondary metabolites, and scaling up the synthesis of valuable plant products under controlled laboratory conditions.

MCQs:

1. What is the first step in the callus culture process?

- a) Culture of shoot tips
- b) Selection of explant
- c) Subculture the callus
- d) Regeneration of shoots

2. What is the significance of callus culture in plant regeneration?

- a) It leads to the formation of roots only
- b) It forms new plant structures from undifferentiated cells
- c) It causes rapid plant growth
- d) None of the above

3. Which process is associated with somatic embryogenesis?

- a) Production of seeds from somatic cells
- b) Production of root-like structures
- c) Formation of organ tissues from callus
- d) None of the above

4. What does micropropagation refer to?

- a) Plant cloning through tissue culture
- b) Producing genetically modified plants



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- c) Direct transplant of plant tissues
- d) In-vitro seed germination

5. Which part of the plant is used in axillary bud culture?

- a) Root
- b) Leaf
- c) Shoot
- d) Seed

6. Which culture method is used for disease-free plant propagation?

- a) Callus culture
- b) Meristem culture
- c) Micropropagation
- d) Both b and c

7. What type of explant is used in shoot-tip culture?

- a) Leaf
- b) Stem
- c) Shoot apex
- d) Root tips

8. What is the role of organogenesis in tissue culture?

- a) Root formation
- b) Shoot formation
- c) Both root and shoot formation
- d) Callus formation

9. What is somaclonal variation?

- a) Genetic variation within a callus population



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- b) Variation in size of the callus
- c) Phenotypic variation in seedlings
- d) None of the above

10. What is the primary purpose of meristem culture?

- a) To regenerate shoots
- b) To produce virus-free plants
- c) To induce root formation
- d) None of the above

Short Questions:

1. Define callus culture.

What are the different types of callus?

3. How does organogenesis differ from somatic embryogenesis?

4. Explain the process of micropropagation.

5. What are the factors affecting callus growth?

6. How is axillary bud culture used in plant propagation?

7. Describe the significance of shoot-tip and meristem culture.

8. What is somaclonal variation and its impact on tissue culture?

9. How does callus culture help in the regeneration of plants?

10. Discuss the role of growth regulators in callus culture.

Short answer type questions:

1. Explain the methods of callus culture and their applications in plant regeneration.

2. Describe the techniques involved in somatic embryogenesis and their advantages.



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3. Discuss the importance of micropropagation in horticulture and agriculture.
4. Explain how axillary bud culture can be used for rapid propagation of plants.
5. Compare and contrast organogenesis and somatic embryogenesis.
6. Discuss the role of shoot-tip culture in the production of disease-free plants.
7. Describe the process of callus formation and differentiation.
8. Discuss the various applications of tissue culture in agriculture.
9. What is the role of growth regulators in cell suspension culture?
10. How do factors like temperature and light affect the growth of callus?



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MODULE 3

INTRODUCTION TO HAPLOID PRODUCTION

Objective:

To understand the production of haploids, their role in plant breeding, and the

techniques used in ovary and anther culture, somaclonal variation, and the in-vitro

production of secondary metabolites.



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Unit 3.1 : Production of Haploid Cells - Ovary and Anther Culture

Haploid cells in plants can be produced through in vitro culture of anthers or ovaries, also known as androgenesis and gynogenesis, respectively. These techniques are crucial for plant breeding and genetics, allowing for the rapid development of homozygous lines.

Androgenesis (Anther/Pollen Culture):

- **Process:**

Androgenesis involves culturing anthers or isolated pollen grains (microspores) on nutrient media to induce them to develop into haploid plants.

- **Mechanism:**

The male gametophyte (microspore) is directed to bypass its normal development into a gamete and instead develop into a haploid plant.

- **Significance:**

Anther culture is a valuable tool for creating doubled haploids (DH), which are essentially pure lines, useful for both self-pollinated and cross-pollinated crops.

- **Advantages:**

Anther culture can accelerate the development of homozygous lines, reducing the time required compared to conventional breeding methods.

Gynogenesis (Ovary/Ovule Culture):

- **Process:**

Gynogenesis utilizes the female gametophyte (ovary or ovule) to produce haploid plants.

- **Mechanism:**

The female gametophyte is stimulated to develop into a sporophyte (a plant with two sets of chromosomes), resulting in a haploid plant.



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- **Significance:**

Gynogenesis is useful when androgenesis is not effective or when there are specific traits that are easier to select for in the female gametophyte.

Mechanism of Ovary Culture

1. Excision and Sterilization:

Ovaries are carefully excised from flowers, ensuring they are free from contaminants. They are then surface sterilized using a disinfectant solution (like ethanol and sodium hypochlorite) and washed with sterile water.

2. Nutrient Medium:

The sterilized ovaries are placed on a nutrient medium, which provides the necessary mineral salts, sugars, vitamins, and growth regulators for growth and development. The specific composition of the medium can vary depending on the plant species and the desired outcome.

3. Induction and Regeneration:

- **Embryogenesis:** In some cases, the ovary tissue can directly differentiate into embryos (embryogenesis) without forming a callus. This is often influenced by the hormonal balance in the medium.
- **Callus Formation:** In other cases, the ovary tissue may form a callus (undifferentiated mass of cells) first, which then differentiates into shoots and roots.

3.1.1 Haploid Production:

Ovary culture can be used to produce haploid plants, particularly through unpollinated ovaries. This process can involve gynogenesis (development of an embryo from the egg cell or other haploid cells within the ovule) or via callus formation and subsequent plant regeneration.

3.1 Plantlet Regeneration:



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Once shoots and roots are formed, the resulting plantlets can be transferred to a fresh medium for further growth and development until they are ready for acclimatization and transfer to soil.

3.1.2 Applications

Ovary culture in plant tissue culture provides a number of useful applications, mostly in researching the early development of the embryo, fruit development, and the generation of haploid plants. Ovary culture provides an opportunity to research the influence of phytohormones on fruit development, including parthenocarpic (seedless) fruit development, and the influence of floral organs on their development. Ovary culture also provides a tool for inducing polyembryony (the development of multiple embryos from one ovary) and researching in-vitro pollination and seed development.

Summary

Haploid production is a plant tissue culture technique aimed at developing plants that contain only a single set of chromosomes (n) instead of the normal diploid set ($2n$). It is primarily achieved through **anther culture, microspore culture, or ovule culture**, where gametophytic cells are induced to develop into haploid plantlets. Haploids are of great importance in plant breeding because they allow the rapid production of **homozygous diploid lines** through chromosome doubling, thus saving several generations of inbreeding. This technique accelerates crop improvement programs, helps in identifying and fixing desirable traits, and provides material for genetic studies. Haploid plants also serve as valuable tools in mutation studies, gene mapping, and hybrid development.



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MCQs on Introduction to Haploid Production

1. Haploid plants contain how many sets of chromosomes?

- a) One set
- b) Two sets
- c) Three sets
- d) Four sets

Answer: a) One set

2. Which of the following is the main objective of haploid production in plants?

- a) Increase heterozygosity
- b) Production of homozygous lines rapidly
- c) Delay breeding programs
- d) To prevent hybridization

Answer: b) Production of homozygous lines rapidly

3. The process of chromosome doubling in haploids to obtain homozygous diploids is called:

- a) Hybridization
- b) Colchicine treatment
- c) In vitro fertilization
- d) Mutation breeding

Answer: b) Colchicine treatment



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4. The first haploid plants were produced in which plant?

- a) Rice
- b) Datura
- c) Tobacco
- d) Maize

Answer: b) Datura

5. Anther culture is mainly used for:

- a) Triploid production
- b) Haploid production
- c) Somatic hybridization
- d) Polyploidy induction

Answer: b) Haploid production

6. Haploid plants are useful in plant breeding because:

- a) They show hybrid vigor
- b) They produce only male gametes
- c) They allow fixation of traits through doubled haploids
- d) They cannot be used for genetic studies

Answer: c) They allow fixation of traits through doubled haploids

7. The method involving culture of microspores for haploid production is called:

- a) Ovule culture



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- b) Anther culture
- c) Microspore culture
- d) Embryo rescue

Answer: c) Microspore culture

8. Which chemical is commonly used to induce chromosome doubling in haploids?

- a) Auxin
- b) Gibberellin
- c) Colchicine
- d) Ethylene

Answer: c) Colchicine

9. In plant tissue culture, haploids can be produced from:

- a) Endosperm
- b) Zygote
- c) Gametophytic tissue
- d) Somatic embryo

Answer: c) Gametophytic tissue

10. The doubled haploid technique is especially useful in:

- a) Animal breeding
- b) Crop improvement programs
- c) Microbial culture
- d) Ornamental plant decoration



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Answer: b) Crop improvement programs

short answer type questions on "Introduction to Haploid Production":

1. What is haploid production in plants?
2. Name two techniques used for haploid plant production.
3. Why are haploids important in plant breeding?
4. What is the difference between haploid and doubled haploid plants?
5. Mention one advantage of haploid production in crop improvement.

Unit 3.2: Somaclonal Variations

Somaclonal variation refers to the genetic and epigenetic changes that occur in plants regenerated from cell and tissue cultures. These changes can lead to variations in traits compared to the original donor plant. While this variation can be undesirable in some applications, like mass propagation, it can also be a valuable tool for plant breeders seeking to introduce new traits or improve existing ones.

3.2.1 Introduction to Somaclonal Variation

Somaclonal variation is the variation seen in plants that have been produced by plant tissue culture. Chromosomal rearrangements are an important source of this variation. The term somaclonal variation is a phenomenon of broad taxonomic occurrence, reported for species of different ploidy levels, and for outcrossing and inbreeding, vegetatively and seed propagated, and cultivated and non-cultivated plants. Characters affected include both qualitative and quantitative traits.



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Somaclonal variation is not restricted to, but is particularly common in, plants regenerated from callus. The variations can be genotypic or phenotypic, which in the latter case can be either genetic or epigenetic in origin. Typical genetic alterations are: changes in chromosome numbers (polyploidy and aneuploidy), chromosome structure (translocations, deletions, insertions and duplications) and DNA sequence (base mutations). A typical epigenetics-related event would be gene methylation.

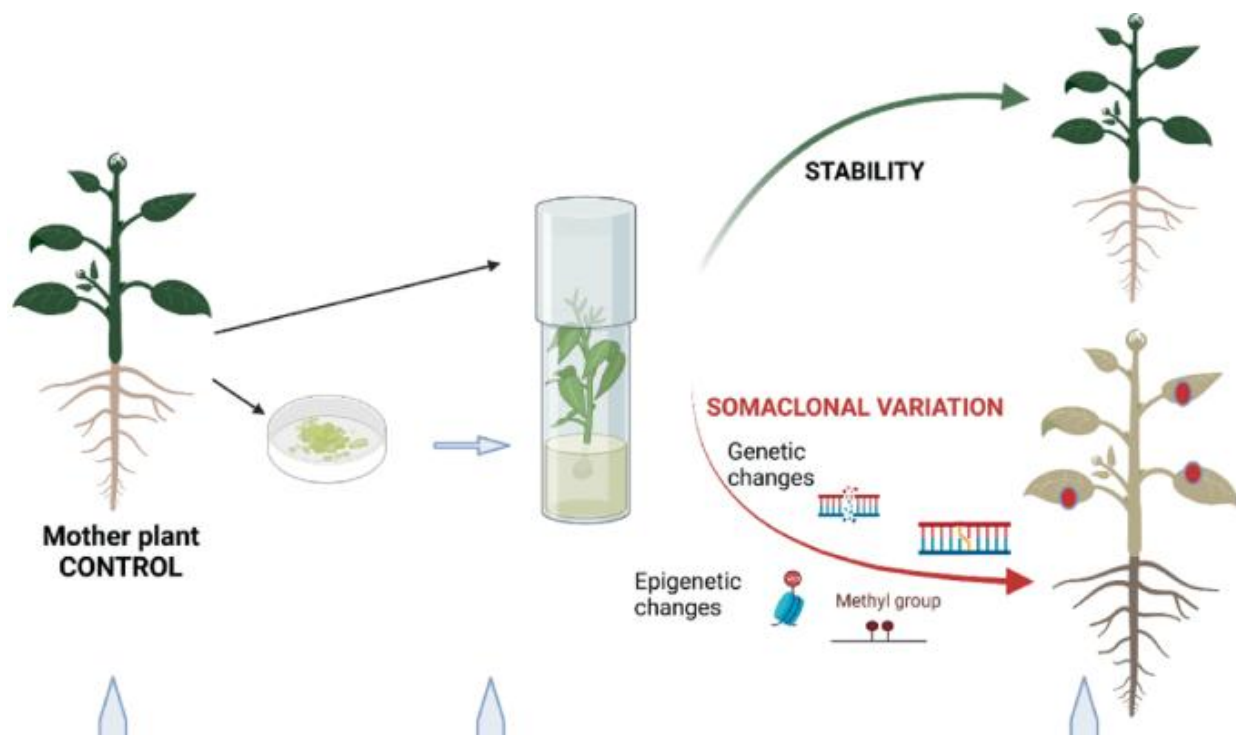
If no visual, morphogenic changes are apparent, other plant screening procedures must be applied. There are both benefits and disadvantages to somaclonal variation. The phenomenon of high variability in individuals from plant cell cultures or adventitious shoots has been named somaclonal variation.

Understanding Somaclonal Variation

Somaclonal variation is defined as genetic and phenotypic variation occurring in plants derived from somatic cells in tissue culture. This phenomenon was also formally recognised and named in a seminal publication by Larkin and Scowcroft in 1981, although researchers had noted observations of unexpected variability in tissue-cultured plants for decades. This term captures all types of variations that are produced during the in vitro culture process, irrespective of the cellular origin of the variant or the particular technique used for plant regeneration. The concept of somaclonal variation was first discovered during the early development of plant tissue culture in the 1950s and 1960s. As plant regeneration techniques improved, it was observed by scientists that regenerated plants occasionally showed phenotypes different from that of the donor plants. More than 40 years ago, these variations were regarded as aberrations that were undesirable because they undermined the genetic uniformity that was to be expected from clonal propagation. But as knowledge about plant genomics progressed, researchers started to realize that this variation could be valued and provide new genetic diversity. Somaclonal variation is highly heterogeneous and covers a

wide range from small biochemical changes to big morphological changes. Variations may appear for almost all plot traits: their height, leaf shape, flowering time, fruit characteristics, pigmentation, growth habit, physiological responses, and so on. The traits of major interest are variations for yield components, and quality and resistance to biotic and abiotic stresses.

Mechanisms of Somaclonal Variation:



- **Karyotypic Changes:**
 - **Polyploidy and Aneuploidy:** Changes in chromosome number (either multiples of the basic set or deviations from the normal number) can occur, leading to variations in gene expression and phenotype.
 - **Chromosome Rearrangements:** Breaks, deletions, inversions, and translocations of chromosome segments can result in altered gene order and function.
- **Gene Mutations:**



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- **Point Mutations:** Changes in the DNA sequence at a single nucleotide can alter protein structure and function.
- **Insertions and Deletions:** Gains or losses of DNA sequences can disrupt gene expression or coding.
- **Epigenetic Modifications:**
 - **DNA Methylation:** Changes in DNA methylation patterns can affect gene expression without altering the DNA sequence itself. These modifications can be heritable but may also be reversed under different conditions.
 - **Histone Modifications:** Modifications to histone proteins, which package DNA, can influence gene expression.
- **Transposable Elements:**

"Jumping genes" or transposable elements can move to new locations in the genome, potentially disrupting gene function or creating new regulatory elements.

- **Physiological Stress:**
 - **Hormonal Imbalance:** Altered hormone levels in the culture medium can induce physiological changes that affect gene expression.
 - **Oxidative Stress:** High levels of reactive oxygen species (ROS) can damage DNA and other cellular components, leading to mutations and other changes.
 - **Wounding and Tissue Injury:** Cellular damage during tissue culture can trigger stress responses that affect gene expression.

Factors Influencing Somaclonal Variation:



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- **Genotype:**

Different plant species and even different varieties within a species can exhibit varying degrees of somaclonal variation.

- **Culture Conditions:**

Factors like the composition of the culture medium, growth hormones, and physical environment can influence the extent of variation.

- **Explant Source:**

The type of tissue used to initiate the culture can affect the frequency and type of variations observed.

- **Duration of Culture:**

Prolonged culture periods tend to increase the likelihood of somaclonal variation.

Factors Influencing Somaclonal Variation:

- **Genotype:**

The genetic makeup of the plant significantly impacts the likelihood and type of somaclonal variation. Different genotypes exhibit varying degrees of susceptibility to variation during tissue culture.

- **Explant Source:**

The specific plant tissue used as the starting material (explant) also plays a crucial role. For example, potato plants regenerated from callus derived from rachis and petiole exhibit a higher frequency of variation compared to those derived from leaf callus.

- **Culture Conditions:**

- **Growth Regulators:** Plant growth regulators like BAP, NAA, and 2,4-D can induce mutations in cultured cells.



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- **Culture Media:** The composition of the culture medium, including growth regulators and other components, can influence the type and frequency of somaclonal variation.
- **Duration of Culture:** Longer culture periods tend to increase the frequency of somaclonal variation.
- **Age of Culture:**

The age of the culture, both the explant and the overall culture duration, can affect the extent of variation.

- **In Vitro Selection:**

The selection methods employed during tissue culture can also influence the types of variants that are recovered.

- **Other factors:**

Other factors like the oxygen gradient, light quality and intensity, temperature, and pH of the medium can also play a role.

3.2.2 Application of Somaclonal Variations

The phenomenon of somaclonal variation that was earlier considered a mere side effect of tissue culture has now become a useful tool with wide applications in plant improvement and germplasm enrichment for development of stress resistant variety and even, fundamental studies of genetics. Due to its spontaneous nature, its capacity to produce new combinations that may not be easily derived from classical hybridization/ genetic methods, and its power to produce variation in genetically uniform backgrounds, somaclonal variation has become one of the mainstays of plant biotechnology programs worldwide. Somaclonal variation is applied most notably in the context of crop improvement. The diversity of genetic and phenotypic composition arising from tissue culture offers plant breeders new calls with traits of agricultural significance. Somaclonal variation, in contrast to



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traditional hybridization, can give rise to new genetic combinations from existing cultivars and potentially preserve the beneficial traits of the cultivar while inducing helpful changes.

Summary

Somaclonal variation refers to the genetic and epigenetic changes that occur in plants regenerated through tissue culture techniques. These variations arise due to chromosomal rearrangements, gene mutations, DNA methylation, or activation of transposable elements during the process of callus formation and regeneration. Somaclonal variation can result in both desirable and undesirable traits. Beneficial variations, such as disease resistance, stress tolerance, improved yield, or enhanced nutritional quality, are often exploited in plant breeding programs. However, unwanted variations can lead to abnormalities in growth, fertility, or morphology. Overall, somaclonal variation provides a valuable source of genetic diversity for crop improvement and complements conventional and molecular breeding methods.

MCQs on Somaclonal Variations

1. Somaclonal variation refers to:

- a) Genetic variation arising from cross-pollination
- b) Variation observed in plants regenerated from tissue culture
- c) Variation due to natural mutations only
- d) Variation induced by chemical mutagens

Answer: b) Variation observed in plants regenerated from tissue culture

2. The term "somaclonal variation" was first coined by:

- a) Murashige and Skoog



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- b) Larkin and Scowcroft
- c) Haberlandt
- d) Skoog and Miller

Answer: b) Larkin and Scowcroft

3. Which of the following is not a cause of somaclonal variation?

- a) Chromosomal rearrangements
- b) Point mutations
- c) Transposable elements
- d) Vegetative propagation by stem cuttings

Answer: d) Vegetative propagation by stem cuttings

4. Somaclonal variation can be considered useful for:

- a) Eliminating genetic variability
- b) Creating disease-resistant plant varieties
- c) Ensuring uniformity in cloned plants
- d) Preventing mutation

Answer: b) Creating disease-resistant plant varieties

5. Which of the following techniques is used to detect somaclonal variation at the DNA level?

- a) ELISA



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- b) PCR-based molecular markers (e.g., RAPD, AFLP, SSR)
- c) Microscopy
- d) Chromatography

Answer: b) PCR-based molecular markers (e.g., RAPD, AFLP, SSR)

6. Somaclonal variation is more frequent in plants regenerated from:

- a) Nucellar embryos
- b) Protoplast cultures and callus cultures
- c) Meristem cultures
- d) Stem cuttings

Answer: b) Protoplast cultures and callus cultures

7. Which crop has benefited significantly from somaclonal variation in developing disease-resistant varieties?

- a) Wheat
- b) Sugarcane
- c) Rice
- d) Banana

Answer: d) Banana

8. Somaclonal variation may involve:

- a) Polyploidy



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- b) Aneuploidy
- c) Gene amplification or deletion
- d) All of the above

Answer: d) All of the above

9. The main disadvantage of somaclonal variation is:

- a) It reduces genetic diversity
- b) It causes unexpected and undesirable traits
- c) It prevents disease resistance
- d) It eliminates hybrid vigor

Answer: b) It causes unexpected and undesirable traits

10. Somaclonal variation is particularly useful in:

- a) Vegetatively propagated crops
- b) Self-pollinated crops only
- c) Cross-pollinated crops only
- d) All monocots only

Answer: a) Vegetatively propagated crops

Short-answer type questions on **Somaclonal Variations:**

1. What is meant by somaclonal variation?



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2. Name two sources of somaclonal variation.
3. How is somaclonal variation induced in plant tissue culture?
4. Mention two advantages of somaclonal variation.
5. Give one example of a crop improved through somaclonal variation.

Unit 3.3 : In-Vitro Production of Secondary Metabolites (Biotransformation)

3.3.1 Introduction to In-Vitro Production of Secondary Metabolites

n-vitro production of secondary metabolites using plant tissue culture involves cultivating plant cells, tissues, or organs in a controlled laboratory environment to synthesize valuable bioactive compounds. This technique offers an alternative to traditional methods of sourcing these compounds from whole plants, potentially leading to increased yields, controlled production, and reduced environmental impact.

Key Aspects of In-Vitro Secondary Metabolite Production:

- **Plant Tissue Culture:**

This technique involves growing plant cells, tissues, or organs (explants) in a sterile, artificial medium under controlled conditions.

- **Secondary Metabolites:**

These are organic compounds produced by plants that are not directly involved in basic growth and development but often play roles in defense mechanisms, attracting pollinators, or other ecological interactions. Examples include alkaloids, flavonoids, terpenoids, and phenolic compounds.

- **Advantages:**



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- **Controlled Production:** In-vitro systems allow for precise control over environmental factors (light, temperature, nutrients, etc.) that influence metabolite production.
- **Increased Yields:** By optimizing culture conditions and using techniques like elicitation (introducing stress factors to stimulate production), in-vitro systems can sometimes produce higher yields of specific metabolites than whole plants.
- **Sustainable Production:** Tissue culture can reduce reliance on wild harvesting, potentially conserving plant biodiversity and reducing environmental impact.
- **Consistent Quality:** In-vitro production can provide a more consistent and reliable source of secondary metabolites compared to harvesting from variable wild populations.
- **Access to Rare Metabolites:** Some metabolites are difficult to obtain in sufficient quantities from natural sources; in-vitro culture can make them accessible.
- **Methods:**
 - **Callus Culture:** Plant cells that have been dedifferentiated are cultured on a nutrient medium to create a mass of non-differentiated cells (callus).
 - **Suspension Culture:** Callus or other plant cells in liquid medium are cultured for mass production.
 - **Hairy Root Culture:** Agrobacterium rhizogenes infection can cause the development of hairy roots, which are renowned for having high metabolite yields.



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- **Elicitation:** Cultures can be treated with elicitors (for example, fungal extracts, certain chemicals) to induce the biosynthesis of secondary metabolites.
- **Challenges:**
 - **Lower Production:** In some cases, metabolite production in-vitro may be lower than in the whole plant.
 - **Genetic Instability:** Cultured cells can be genetically unstable, leading to variations in metabolite production over time.
 - **Scale-up Issues:** Scaling up in-vitro production to industrial levels can be challenging and costly.
 - **Aseptic Conditions:** Maintaining sterile conditions is crucial to prevent contamination, which can negatively impact production.
- **Applications:**
 - **Pharmaceuticals:** Many secondary metabolites have medicinal properties and are used in drug development.
 - **Food Industry:** Some metabolites are used as food additives, flavorings, or colorants.
 - **Cosmetics:** Secondary metabolites are used in cosmetic products for their various properties.
 - **Agriculture:** Some metabolites can be used as natural pesticides or herbicides.
- **Future Directions:**
 - **Metabolic Engineering:** Researchers are using genetic engineering techniques to enhance metabolite production pathways in plant cells.



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- **Bioreactors:** Large-scale bioreactors are being developed to optimize metabolite production in suspension cultures.
- **Nanotechnology:** Nanomaterials are being explored to improve metabolite extraction and delivery.

Biotransformation in Secondary Metabolite Production

Biotransformation in plant tissue culture refers to the enzymatic conversion of a precursor compound into a more valuable product by plant cells or enzymes. This process is particularly useful for producing secondary metabolites, which are often complex molecules with medicinal or industrial applications. Plant cell cultures can be engineered to perform specific biotransformation reactions, leading to the production of novel compounds or enhanced yields of known metabolites.

3.4.2 Key aspects of biotransformation in plant tissue culture:

- **Enzymatic conversion:**

Biotransformation relies on the catalytic activity of enzymes present in plant cells to modify precursor molecules.

- **Precursor compounds:**

These can be natural intermediates in a biosynthetic pathway or synthetic compounds introduced into the culture.

- **Secondary metabolites:**

Biotransformation is commonly used to produce diverse secondary metabolites, including alkaloids, terpenoids, and phenolics.

- **Applications:**

Biotransformation in plant tissue culture has applications in pharmaceuticals, cosmetics, and other industries.

- **Advantages:**



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It can offer advantages over traditional chemical synthesis, such as regio- and stereoselectivity, and the ability to produce complex molecules efficiently.

3.3.3 Complicated extraction and purification process

Plant tissue culture extraction and purification processes can be quite complex, involving multiple steps to ensure successful isolation and purification of specific plant compounds. These processes are crucial for various applications, including the production of valuable secondary metabolites and genetic research.

Key steps in plant tissue culture extraction and purification:

1. 1. Explant Selection and Sterilization:

- Healthy plant tissue (explants) are selected and surface-sterilized to eliminate microbial contamination. This step is vital as contamination can hinder the entire process.
- Various methods, including chemical sterilization with alcohol and bleach, are employed. The choice of method depends on the explant's sensitivity and the level of contamination.

2. 2. Culture Media Preparation:

- Appropriate nutrient media, containing inorganic salts, vitamins, plant hormones, and sometimes amino acids, are prepared.
- Solid media is prepared by adding a gelling agent like agar.

3. 3. Culturing and Scaling Up:

- Sterilized explants are placed in the prepared media under controlled environmental conditions (temperature, light, humidity).



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- Depending on the desired outcome (e.g., micropropagation, callus culture), the cultures are scaled up through repeated subcultures.
- Bioreactors may be used for large-scale culture of callus and cell suspensions.

4. Extraction:

- **Solvent Extraction:** Various solvents (e.g., ethanol, hexane, ethyl acetate) are used to extract desired compounds from the cultured plant tissues.
- **Other Methods:** Other techniques like steam distillation, cold pressing, and supercritical fluid extraction may also be used depending on the nature of the target compound.

5. Purification:

- **Chromatography:** Techniques like column chromatography (e.g., silica gel, HPLC) and thin-layer chromatography are used to separate and purify the extracted compounds based on their physical and chemical properties.
- **Recrystallization:** This method is used to further purify compounds by dissolving them in a solvent and then allowing them to crystallize out, leaving impurities behind.
- **Other Methods:** Other methods, such as membrane filtration and liquid-liquid extraction, may also be employed depending on the specific compound and its properties.

6. Analysis and Characterization:

- Purified compounds are analyzed and characterized using techniques like spectroscopy (e.g., UV-Vis, IR,



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NMR) and mass spectrometry to confirm their identity and purity.

7. Downstream Processing:

- Purified compounds are formulated, packaged, and prepared for their intended application.

Lowered risk of contamination by toxic compounds

The development of in-vitro methodologies for the production of secondary metabolites implies a convergence of several scientific fields such as plant physiology, microbiology, biochemistry, molecular biology and bioprocess engineering. Success with plant tissue cultures began in the 1950s and 1960s with study of their production of secondary metabolites, although often still at lower levels than might be found in intact plants. The subsequent decades ushered advances in elucidating the biosynthetic pathways and regulatory mechanisms of secondary metabolism, development of elicitation approaches to achieve increased production and utilization of genetic engineering tools to modify secondary metabolic pathways. At present, in-vitro production of secondary metabolites involves a few main methods:

- Plant cell and tissue culture systems (callus, cell suspension, hairy root, and shoot cultures)
- Bacteria, yeasts, and filamentous fungi microbial fermentation systems
- Systems based on isolated or immobilized enzymes
- Transgenics, genetically modified organisms
- Combinatorial biosynthesis strategies

Methods of In-Vitro Production of Secondary Metabolites

In-vitro production of secondary metabolites in plant tissue culture involves techniques like cell suspension cultures, hairy root cultures,



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and immobilized cell cultures to cultivate plant cells and produce desired compounds under controlled conditions. These methods allow for manipulation of growth conditions and elicitation to enhance metabolite production.

Key Methods:

- **Callus Culture:**

Callus, an undifferentiated mass of plant cells, is induced from explants (e.g., leaf, stem) and can be used to initiate cell suspension cultures or for micropropagation.

- **Cell Suspension Cultures:**

Plant cells or callus are grown in liquid medium, allowing for large-scale cultivation and metabolite production.

- **Hairy Root Cultures:**

Plant cells transformed with *Agrobacterium rhizogenes* develop into hairy roots, which can be cultured in vitro to produce specific secondary metabolites.

- **Immobilized Cell Cultures:**

Plant cells or aggregates are trapped or attached to a support matrix, allowing for continuous metabolite production while minimizing shear stress.

- **Elicitation:**

This involves applying external stimuli (elicitors) to the plant cells to induce or enhance the production of secondary metabolites. Elicitors can be biotic (e.g., fungal extracts, yeast extract) or abiotic (e.g., methyl jasmonate, salicylic acid).

- **Medium Optimization:**

Adjusting the composition of the culture medium (e.g., nutrient levels, hormone concentrations) to promote cell growth and metabolite production.



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- **Environmental Control:**

Manipulating factors like light, temperature, and aeration can influence metabolite synthesis.

- **Biotransformation:**

Using plant cells to convert precursor molecules into desired secondary metabolites.

- **Metabolic Engineering:**

Altering the metabolic pathways within plant cells to enhance the production of specific secondary metabolites.

Steps Involved:

- 1. Selection of Plant Material:**

Choosing the right plant species or cell lines known for producing the desired metabolites.

- 2. Callus Induction and Maintenance:**

Initiating callus culture from plant explants and maintaining it under appropriate conditions.

- 3. Cell Suspension Culture Development:**

Establishing cell suspension cultures from callus for large-scale production.

- 4. Optimization of Culture Conditions:**

Adjusting medium composition, elicitor application, and environmental factors to maximize metabolite production.

- 5. Harvesting and Purification:**

Extracting and purifying the desired secondary metabolites from the culture.

Reactions of condensation and addition

Dehalogenation, dealkylation deamination



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several of these transformations are catalyzed by classes of enzymes. For example, hydroxylation reactions are commonly performed by cytochrome P450 monooxygenases, and glycosyltransferases introduce a sugar moiety to metabolites yielding often more soluble and stable derivatives.

Scale of production needed

Substrate cost, growth medium, and downstream processing are some of the economic factors that must be considered while keeping in view economic factors. Biotransformation possesses one main application like enhancement of the bioactivity or bioavailability of natural compounds. Numerous secondary metabolites in their natural state present poor pharmaceutical application due to low solubility, stability or bioavailability. This limitation can be overcome by biotransformation of both small molecules and large molecules where selective modifications can be used (e.g. glycosylation, hydroxylation, methylation). Fungal biotransformation to more active paclitaxel derivatives has enhanced its anticancer activity, for instance. Detoxification of plant secondary metabolites is another important application. Most toxins from plants are defense mechanisms, and they can be detoxified through biotransformation by producing non-toxic or less toxic derivatives. Cassava cyanogenic glycosides and various mycotoxins have been studied and reported to operate in this modulation. Biotransformation is also an appropriate tool for drug discovery and development. It expands the chemically accessible space that can be screened and may yield lead compounds with more favorable pharmacologic properties by creating new derivatives. This procedure is typified by the antibiotic erythromycin and its numerous semi-synthetic derivatives produced by biotransformation. Although biotransformation has several strengths for secondary metabolite generation, it is confronted with several major challenges:

Narrow substrate specificity of some biological systems



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- Limited stability of some substrates or products in biological environments
- Toxicity of substrate or product to the biotransforming organism
- Limited transformation with low conversion rates
- Acceleration of scale-up from lab to plant.

Challenges related to downstream processing

Research is already being done to tackle these obstacles, with strategies like directed

evolution of the enzymes used, metabolic engineering of host organisms, process

optimization and unique design of bioreactors. Biotransformation can be coupled with

other technologies, like immobilized cell systems and in-situ product recovery, making

it more suitable for secondary metabolite production.

In-vitro Techniques to Produce Secondary Metabolites

While the in-vitro production of secondary metabolites covers different types of methodologies with its unique features with each type having its advantages and

disadvantages. These include cell and tissue culture-based systems, enzyme-based

methods, and genetically engineered systems. Selecting an appropriate method depends

on the specific metabolite, the biosynthetic complexity and the desired scale of

production.



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Plant Cells and Tissues:

Culture Systems

Among in-vitro methods, plant cell and tissue culture is one of the oldest and most commonly used methods for the production

of secondary metabolites. This approach makes use of the totipotency of plant cells

capable of specialization into cell types as well as the possibility of whole plant

regeneration. This category includes multiple different culture systems:

Callus culture:

Callus is an unorganized mass of undifferentiated cells started from the explants (plant tissue fragments) on solid media with proper plant growth regulators.

While some secondary metabolites are produced by callus cultures themselves, others,

however, are starting points for establishment of other culture systems. Examples are

represented by the callus cultures of *Catharanthus roseus*, which produce alkaloids,

and those of *Taxus* species producing taxoids like paclitaxel. But callus cultures yield

lower concentrations of metabolites than those of the source plants as the cultures lack

tissue differentiation and specialized storage structures.

Cell Suspension Cultures:

Cell suspension cultures are formed from friable callus,



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which is a group of cells/the tissue in small cells aggregates growing in shaking liquid

media. It provides benefits, including faster growth rates, improved homogeneity.

Physical Factors

3.4.4 Light (Intensity, Quality, and Photoperiod):

Light can be regarded as an energy

source and a regulatory signal that modulates secondary metabolism. For primary metabolism, cultures need suitable light that subsequently also favor secondary metabolites production, albeit indirectly. More immediately, light controls many biosynthetic pathways via photoreceptors such as phytochromes and cryptochromes. Anthocyanin biosynthesis has been shown to be increased in many plant cultures at high light intensities, in particular blue light significantly stimulated rosmarinic acid accumulation in *Coleus* cultures. On the other hand, some alkaloids bubbles preferentially accumulate in total darkness. Rhythmic metabolic processes are also affected by photoperiod (light/dark cycling). By controlling these factors, production can be adjusted in photosensitive systems.

Kinetics:

Temperature impacts growth rates and flux distributions metabolic. Plant cell cultures generally show the best growth at 24–28 °C, whereas fungal cultures should be grown at 25–30 °C and bacterial cultures at 28–37 °C; optimum growth temperatures, however, are usually not the same as optimum secondary metabolite production temperatures. In several plant culture systems, the accumulation of metabolites has been enhanced by using sub-optimal growth temperature—e.g., ginsenoside production in *Panax ginseng* increased by otherwise growth-limiting temperatures (20°C); shikonin production in *Lithospermum* was promoted at high temperatures (30°C). Temperature fluctuations tend



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to induce metabolic responses favorable for secondary metabolism that is exploited for two-phase culture systems.

Gaseous Environment (O_2 , CO_2):

The availability of oxygen plays a critical role in determining aerobic cultures' primary and secondary metabolism. The growth and production of high-density cultures are limited by insufficient oxygen transfer, while excessive aeration can induce oxidative damage and degradation of metabolites. Safety of high dissolved oxygen concentration depends on organism and targeted metabolite 30–50% oxygen saturation used for penicillin production and it is well known that some plant metabolites accumulate more efficiently under mild hypoxia. Carbon dioxide concentration also impacts metabolisms, and in photosynthetic cultures, higher CO_2 can often boost biomass production while negatively affecting the accumulation of secondary metabolites and compound productivity. The correct balance of these gases through well-optimized aeration, agitation and bioreactor design is critical to maximizing production.

Chemical Factors

Nutrient Composition (Macro and Microelements):

The composition of the culture media is one of the key factors that affect growth and secondary metabolism. Carbon sources (usually sugars) provide energy and carbon skeletons, with source

type and concentration having a sizeable influence on production, e.g., glucose contributes to anthocyanin production in many plant cultures, while sucrose increases alkaloid biosynthesis. There also be nitrogen sources (e.g., inorganic salts or organic compounds) that have similar effects on metabolism for instance, nitrate-to-ammonium ratios that shift pH and metabolic pathways. Phosphate serves both as nutrient and regulatory molecule and high concentrations of phosphate can repress secondary metabolism through repression mechanisms. Enzyme cofactors and regulatory signals, thereby linking signaling pathways,



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the particular roles of which are discussed here, together. The nutrient composition must be systematically optimized, for each production system.

Plant Growth Regulators and Hormones:

Dramatic effects on secondary metabolism in plant-based systems by plant growth regulators. Auxins and cytokinins, which are crucial for growth and differentiation, exhibit variable effects on metabolite production; for example, 2,4-D induced anthocyanin production in some cultures while inhibiting alkaloid synthesis in others. Abscissic acid typically enhances secondary metabolism simulating stress conditions, whereas gibberellins have variable effects. Additionally, in microbial systems, quorum-sensing molecules and other signaling metabolites enact density-dependent gene regulation of secondary metabolism. The ideal mix of hormones can differ based on the target metabolite and culture system, requiring empirical fine-tuning.

pH of the Medium:

The medium pH has an effect on enzyme functions, nutrient availability and cell membrane permeability. Plant cell cultures generally grow under slightly acidic conditions (pH 5.5-6.0), bacterial cultures in near-neutral (pH 6.8-7.2) and fungal cultures in acidic (pH 4.5-5.5). During cultivation, pH may change because of preferential uptake of nutrients, the secretion of organic acids, or metabolic activity. These changes can also affect secondary metabolism—alkaloidal transport across membranes is driven by pH gradients, and the enzyme activities in phenylpropanoid pathways show pH optima. Buffering or controlled adjustment of pH is important for steady production.

Oxygen Radicals and Oxidative Stress:

ROS Can Be Both Damaging Agent and Signaling Molecule. Moderate oxidative stress usually induces secondary metabolism in the stress response, whereas high ROS levels lead to cell damage. Controlled addition of hydrogen peroxide or ROS-generating compounds can



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sometimes enhance production—peroxide treatment enhances taxol production in *Taxus* cultures, oxidative stress increases alkaloid production in a number of systems. But secondary metabolites themselves often have antioxidant activity, complicating feedback processes with respect to culture redox status.

Summary

Haploid production is a vital technique in plant biotechnology that involves the generation of plants with a single set of chromosomes (haploids) instead of the usual two sets (diploids). This method accelerates the process of developing homozygous lines, which are essential for plant breeding and genetic studies. Haploids can be produced through various methods such as anther culture, pollen culture, and ovule culture, often followed by chromosome doubling to produce doubled haploids. These doubled haploids are genetically stable and uniform, making them valuable for crop improvement, hybrid seed production, and gene mapping. Haploid technology plays a significant role in reducing the time and resources needed for conventional breeding programs.

Summary on Introduction to In-Vitro Production of Secondary Metabolites

In-vitro production of secondary metabolites refers to the synthesis of bioactive compounds by plant cells, tissues, or organs cultured under controlled laboratory conditions. Secondary metabolites such as alkaloids, flavonoids, terpenoids, and phenolics are not directly involved in plant growth and development but play a crucial role in defense, adaptation, and medicinal applications. Conventional extraction of these metabolites from whole plants is often limited due to low yield, long growth cycles, and environmental dependence. Plant tissue culture techniques, including callus culture, cell suspension



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culture, organ culture, and hairy root culture, provide a sustainable and efficient alternative for metabolite production. These systems can be optimized by using elicitors, precursors, and bioreactors to enhance metabolite accumulation. In-vitro methods thus offer a reliable approach for large-scale production of pharmaceutically and industrially important compounds, reducing pressure on natural resources and ensuring continuous supply of high-value products.

MCQs:

1. What is the main goal of haploid production in plants?

- b) Achieve genetic uniformity
- c) Produce disease-resistant plants
- d) Increase size of the crop

2. Which plant part is typically cultured to produce haploids?

- a) Root
- b) Leaf
- c) Anther
- d) Stem

3. What is somaclonal variation?

- a) Genetic variation caused by somatic cell division
- b) Variation in the environment
- c) The mutation rate during pollination



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d) Changes due to natural selection

4. What is the role of anther culture in haploid production?

a) It induces root formation

b) It produces pollen from somatic cells

c) It generates male gametes for fertilization

d) It regenerates a whole plant from anther cells

5. Which process involves the conversion of secondary metabolites

into useful compounds?

a) Biotransformation

b) Cloning

c) Micropropagation

1. d) Germination

2. **What is the main benefit of somaclonal variation in plant breeding?**

a) It helps to develop new varieties with desirable traits

b) It increases plant regeneration rate

c) It helps in in-vitro propagation

d) It prevents diseases

3. **What is an important factor affecting the production of secondary**

4. **metabolites in-vitro?**

a) Hormone concentration

b) Temperature



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- c) Light conditions
 - d) All of the above
5. **Which of the following is a method of in-vitro production of**
6. **secondary metabolites?**
- a) Callus culture
 - b) Somatic embryogenesis
 - c) Organogenesis
 - d) All of the above
7. **What is the major application of haploid production in agriculture?**
- a) Seedless fruit production
 - b) Rapid genetic improvement
 - c) Increased photosynthesis Enhanced drought resistance
8. **10. In which of the following scenarios is somaclonal variation most**
9. **likely to occur?**
- a) Tissue culture propagation
 - b) Natural plant breeding
 - c) Pollination
 - d) Controlled crossbreeding
10. **Short Questions:**
- 1. Define haploid production.
 - 2. Explain the process of ovary culture.
 - 3. What are the advantages of haploid plants in breeding?
 - 4. Describe the significance of anther culture in plant breeding.
 - 5. What is somaclonal variation and how is it useful in agriculture?



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6. Explain the role of biotransformation in secondary metabolite production.
7. How do light and temperature affect the production of secondary metabolites?
8. What are the methods of inducing somaclonal variation?
9. Describe how secondary metabolites are produced in plant tissue culture.
10. Discuss the importance of haploids in crop improvement.

MODULE 4

INTRODUCTION TO PROTOPLAST CULTURE

Objective:

To study the techniques and applications of protoplast culture, including protoplast

isolation, fusion, somatic hybridization, and cybrids.

Unit 4.1 Protoplast Culture – Isolation, Regeneration, and Viability Test



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4.1.1 Introduction to Protoplast Culture

Protoplast culture is emerging as one of the advanced methods of plant biotechnology and lies at the root of genetic manipulation and crop improvement. The isolation of protoplasts, or plant cells without cell walls through the mechanical method on tomato root tips, was first successfully carried out in 1960 by Cocking (1960); however, plant cells without their cell wall remained in division for a short period and they did not survive outside of tissue for long periods of time. This was a major advance in cellular study that also showed how plastic plant cells could really be. Protoplasts hold great utility because of their totipotency (ability to regenerate intact plants) as well as their ease of genetic manipulation followed by regeneration (cell wall makes it hard if not interfere otherwise). Protoplast technology has advanced considerably over the next decades, becoming from a laboratory curiosity to an important tool in modern agricultural biotechnology. The protoplast has been of interest to scientists since the beginnings of cell biology, as it is the basic unit of cellular living. Even the term “protoplast,” from the Greek, refers to the living material of the cell, exclusive of the cell wall. In plants, this includes the plasma membrane, cytoplasm, nucleus, and several organelles. The true value of protoplasts lies in their unique physiological state a plant cell that has temporarily shed its protective but limiting cell wall, which presents the plasma membrane directly to the environment. Being in this exposed state allows the introduction of various manipulations, such as foreign genetic material, organelles and even whole cells introduced via fusion methods. Protoplast culture has a range of applications in scientific and practical fields. In fundamental research, protoplasts are great models to study cellular processes, membrane function, and cell behavior to environmental signals.

Isolation of Protoplasts

The isolation of protoplasts is an indispensable first step in protoplast culture technology; it demands a finely-tuned combination of



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enzymatic digestion and mechanical manipulation to free intact living protoplasts from their wall confines while preserving their MMA. There are principally two methods for protoplast isolation, a mechanical method and an enzymatic method; although a mechanical method has largely been replaced by an enzymatic method because of its better efficiency and gentler handling of cellular components. Klercker (1892) devised the mechanical method, where protoplasts are released through plasmolysis after cutting plant tissues. This method, although landmark in history, provides few protoplasts and causes significant cellular damage. In comparison, the introduction of the enzymatic method by Cocking in 1960 revolutionized protoplast isolation.

Factors Affecting Protoplast Isolation

The successful isolation of protoplasts that are both viable and in sufficient numbers is a complex process, influenced by a variety of factors. These factors are broadly classified into the plant material, enzymatic treatment, and physical factors during isolation. Knowing about the manipulation and optimization of these variables are key steps for establishing effective protocols adapted to particular plant species and research goals. In fact, the attributes of the source plant material dramatically influence the efficacy of protoplast isolation. Plant species show great variation in amenability to protoplast isolation, with some yielding high numbers of viable protoplasts while others being surprisingly recalcitrant even after significant modifications to protocols. This variability is primarily due to differences in composition and structure of plant cell walls with a greater proportion of pectin or lower lignification tend to allow easier release of protoplasts. In fact, even a single species of bacteria may exhibit different responses to the same protocols of isolation leading to the necessity for optimization specific to particular genotypes. Additionally, the physiological state of the plant material makes the isolation challenging. Young and actively growing tissues are expected to produce more viable protoplasts than mature or senescent tissues,



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since they have less rigid cell walls and a higher metabolic activity. Specifically, those plants are more likely to grow under controlled environmental conditions and with reduced light intensity that leads to the development of thinner and easily penetrable cell walls that facilitate enzyme penetration and protoplast release. Successes with isolation can vary from season to season with many researchers reporting best results during specific times of growth. Another key factor in the efficacy of isolation is the developmental nature of the source tissue. Meristematic and embryonic tissues generally provide smaller protoplasts with better sign and is difficult to isolate because of less bulkiness. By contrast, highly vacuolated cells of expanded leaves yield larger numbers of larger protoplasts that are potentially less regenerative. Pretreatment of the plant material before enzyme digestion could considerably increase protoplast yield . Common pretreatment methods include cold shock, plasmolysis in hypertonic solutions, vacuum infiltration of enzyme mixtures, and mechanical scoring or sliced tissue to facilitate access to the enzyme. For otherwise recalcitrant tissues, preincubations with cell wall– loosening agents like pectinase or treatments with growth regulators altering the wall properties may help with the subsequent release of protoplasts. Enzymatic treatment is central to protoplast isolation protocols, and needs careful optimization through a multitude of variables. Choosing suitable enzymes with activity spectra that are aligned with the composition of the target cell wall remains a key consideration As commercial enzyme preparations differ greatly in their degree of purity, activity, and efficiency on different plant substrates, empirical testing is required to determine the best formulations. The concentrations of the individual components in the enzyme mixture need to be carefully optimized—too high an enzyme concentration leads to rapid protoplasts release accompanied by a non-specific degradation of cellular components resulting in poor viability, whereas insufficient enzyme concentrations lead to incomplete digestion and low yields. The length of enzymatic treatment also requires careful balancing, as long incubations can contribute to yield at the expense of viability.



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Separation of the two events temporally has resulted in an increasing number of researchers utilizing sequential enzyme applications, or pulsed exposure protocols for the purpose of maximizing yield while maintaining quality.

Sorting of Protoplasts Based on Viability

The assessment of isolated protoplast viability is a crucial part of protoplast culture protocol, as it gives information about preparation quality and their ability for successful regeneration or experimental use. Viability testing is a set of techniques designed to differentiate living, functional protoplasts from damaged or dead ones, so researchers can assess isolation protocols, predict the outcome of a culture, and determine further downstream applications. These techniques vary from basic microscopic evaluations to advanced biochemical tests, all of which have specific benefits and drawbacks. The simplest viability assay methods are morphological assessment under a light microscope. They should be spherical in shape, and should have highly visible internal cell structures, such as the chloroplasts of photosynthetic tissues — which should be full and bright green — should be clearly observed. The plasma membrane should form a smooth and continuous boundary visible ruptures or blebs. Grouping them all in one aim to trigger cytoplasmic streaming which is detectible as movement of organelles and particles within the protoplast itself as a sign of overall metabolic activity and membrane integrity. Amidst all these, this qualitative assessment, although subjective and useful only for people with relevant experience to interpret correctly, does provide a prompt feedback devoid of any further interventions or specialised equipment. Dye exclusion tests utilize the selective permeability of intact plasma membranes to discriminate between viable and non-viable protoplasts. The most widely used vital stain, fluorescein diacetate (FDA), passes across plasma membranes of living cells where intracellular esterases cleave the molecule releasing fluorescein which becomes trapped within viable protoplasts because of its charged nature. Under fluorescence microscopy, these viable protoplasts present bright green



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fluorescence, while dead cells are non-fluorescent. This method gives a clear visual distinction and can even be quantified by enumerating fluorescent protoplasts vs nonfluorescent ones. Other vital stains include calcein-AM, which acts like FDA but has better retention properties, and carboxy-DCFDA, which provides greater photostability. By contrast, exclusion dyes like Evans blue, trypan blue, or propidium iodide only pass through disturbed membranes, selectively staining non-viable protoplasts. These complementary strategies, critical staining and exclusion staining, can be used side by side in dual staining protocols that allow for discernible distinction of viable, compromised, and non-viable protoplasts. Metabolic activity assays offer a more functional assessment of protoplast viability beyond membrane integrity. Reduction of tetrazolium salts (particularly 2,3,5-triphenyltetrazolium chloride (TTC), but more sensitive ones include MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)) by mitochondrial dehydrogenases in viable cells generates colored formazan crystals which are visible under the microscope, or can be measured spectrophotometrically. Similarly, the bioconversion of resazurin (Alamar Blue) to fluorescent resorufin by metabolically active cells provides a highly sensitive non-toxic viability indicator for the timely tracking of protoplast populations.

Plant Regeneration from Protoplasts

Whole-plant regeneration from isolated protoplasts exemplifies the state of the art of the protoplast culture technology, illustrating the remarkable totipotent nature of plant cells, and offers applications in various fields of plant improvement and research. This entails a bidirectional cascade of developmental phases, from wall reconstruction through initial division to organogenesis and plant establishment, all of which depend on environmental settings and tightly controlled growth regulators. Ineffable protoplast regeneration success fluctuates, considerably, with one species easily giving rise to new individuals while others achieve partial or complete recalcitrance, easily suffering from myriad methodology refinements. The



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regenerative process starts with cell wall reconstruction, which is a crucial first step that converts weak cell protoplasts into walled cells that can continue to divide over the long-term. This process starts within hours of isolation, as cellulose microfibrils are deposited onto the surface of the plasma membrane by membrane-associated cellulose synthase complexes. Hemicelluloses, pectins, and structural proteins are deposited in a second stage, generating a functional, although initially thin and incomplete, primary wall. Not only does the reconstitution of the cell wall complex have structural implications, but it also conveys the dynamic capabilities of polarized growth, intercellular communication, and responses to morphogenic signals. Initially, the makeup and architecture of the newly formed wall differs from those observed in native walls, eventually normal characteristics are assumed as development proceeds. Throughout this regeneration period of the wall, osmotic conditions need to be suitable—usually supplied by culture media containing mannitol, sorbitol or glucose, at concentrations that should not rupture protoplasts but which allow for osmodynamics adjustment in due course as the wall is forming. After having reconstructed a sufficient portion of the wall, protoplast-derived cells transition into division and proliferative states where sustained mitotic activity yields multicellular microcolonies. The beginning of this division is usually the biggest hurdle in regeneration protocols, especially for recalcitrant species. The first division usually takes place 2- 7 days after isolation, although this depends on the species, tissue of origin, and culture conditions employed. Auxins (e.g. dichlorophenoxyacetic acid, naphthaleneacetic acid, or indole-3-acetic acid) are key players in this respect, and a variety of other hormones and relative concentrations in the culture medium can impact and/or induce division. Cytokinins (e.g. benzylaminopurine, kinetin, or zeatin) are often used in conjunction with auxins, with relative concentrations and specific combinations requiring empirical optimization for each system. Nurse cultures, in which protoplasts are cocultured with actively proliferating cells separated by a membrane or as feeder layers, have also been shown to increase rates of division, as conditioning



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factors secreted by surrounding cells promote progression through the cell cycle.

Next-generation antibiotics: prospects and conclusions

Protoplast culture technology has developed from a laboratory curiosity to a powerful biotechnological tool that can be applied in a range of plant science and crop genetic improvement applications, with significant advances made in protoplast isolation, viability testing and regeneration. Transforming intact plant cells into wall-less protoplasts offers exciting opportunities for genetic manipulation and cellular analysis, representing a process with fascinating plasticity and resilience on behalf of plant cells. Although there have been notable advancements in improving methodologies, and extension to many of the plant species, there are still challenges to address the standardization of protocols, low regeneration frequency for recalcitrant species and reduced somaclonal variation in regenerated tissues. Just like protoplast technology, the future seems bright for the former and here are few developmental trends that may enhance or broaden the scope of protoplast technology. Novel imaging technologies, as high-resolution microscopy and non-invasive fluorescence-based methods, can provide unique information on protoplast behavior during the isolation, culture and regeneration phases. When coupled with single-cell -omics tools, these visualization approaches offer thorough assessment of cellular responses to protoplast manipulation and the discovery of determinants for successful regeneration. e.g., CRISPR-Cas systems are proven genome editing tools that promise to expand the toolbox based on protoplasts for crop-oriented target generation while eliminating the need for a costly transformation process. Automation and high-throughput methods for protoplast isolation and culture will allow research and applications to be accelerated by increasing throughput and thereby reducing variability.



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Summary

Protoplast Culture involves the isolation and cultivation of plant cells without their cell walls, enabling studies in cell biology, genetic engineering, and somatic hybridization.

Isolation: Protoplasts are obtained by removing the cell wall either mechanically or enzymatically. Enzymatic methods use cellulase, pectinase, or macerozyme to digest the wall. The quality of protoplasts depends on factors like plant tissue source, enzyme concentration, pH, osmoticum, and incubation time.

Regeneration: Once isolated, protoplasts are cultured in an osmotic stabilizing medium to prevent bursting. Under suitable conditions, they can regenerate a new cell wall, undergo cell division, form callus, and eventually develop into whole plants. This process is critical for applications in transgenic plant development and somatic hybridization.

Viability Test: To assess whether isolated protoplasts are alive and functional, viability tests are performed. Common methods include using vital stains such as fluorescein diacetate (FDA), which fluoresces in live cells, and Evan's Blue, which stains dead cells. Viable protoplasts show intact membranes, metabolic activity, and the ability to divide.

MCQs

Protoplasts are obtained by removing which structure from plant cells?

- a) Plasma membrane
- b) Cell wall
- c) Nucleus
- d) Chloroplast

Answer: b) Cell wall



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Which enzyme is commonly used for the degradation of cellulose in protoplast isolation?

- a) Pectinase
- b) Cellulase
- c) Amylase
- d) Protease

Answer: b) Cellulase

The combination of cellulase and pectinase enzymes helps in:

- a) Breaking the plasma membrane
- b) Removing cell wall completely
- c) Stimulating regeneration
- d) Increasing chlorophyll content

Answer: b) Removing cell wall completely

Osmotic stabilizers used in protoplast isolation include:

- a) NaCl and HCl
- b) Mannitol and Sorbitol
- c) Glucose and Sucrose
- d) Urea and Glycerol

Answer: b) Mannitol and Sorbitol

Which method is commonly used to test the viability of protoplasts?

- a) TTC reduction test



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- b) Evans blue dye exclusion test
- c) Iodine test
- d) Gram staining

Answer: b) Evans blue dye exclusion test

Which culture medium is generally used for protoplast culture?

- a) LB medium
- b) Murashige and Skoog (MS) medium
- c) Nutrient broth
- d) YPD medium

Answer: b) Murashige and Skoog (MS) medium

Fusion of isolated protoplasts can be induced by:

- a) PEG (Polyethylene Glycol) and Electrofusion
- b) Only PEG
- c) Only calcium chloride
- d) Heat shock alone

Answer: a) PEG (Polyethylene Glycol) and Electrofusion

During protoplast regeneration, the first step is:

- a) Division of the nucleus
- b) Cell wall synthesis
- c) Chloroplast formation
- d) Organ development



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Answer: b) Cell wall synthesis

Which factor greatly influences the success of protoplast regeneration?

- a) Light intensity only
- b) Source of explant and growth regulators
- c) Temperature alone
- d) Only medium pH

Answer: b) Source of explant and growth regulators

The technique of protoplast culture is widely used in:

- a) Production of artificial seeds
- b) Somatic hybridization
- c) Micropropagation by nodal culture
- d) In situ hybridization

Answer: b) Somatic hybridization

short answer type questions

- What is a protoplast?
- Name two enzymes commonly used for protoplast isolation.
- What is the role of cellulase in protoplast isolation?
- Why is osmoticum important during protoplast isolation?
- Mention one mechanical and one enzymatic method of protoplast isolation.

Unit 4.2 : Somatic Hybridization

Introduction to Somatic Hybridization



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Somatic hybridization is one of the most important advances in the field of plant biotechnology that allows the fusion of somatic cells (most somatic cells are diploid) of plants from different species to create new/unusual hybrid. This technique was established in the 1970s when researchers started searching for methods to overcome the hurdles of conventional sexual hybridization that is often limited by reproductive incompatibility between distantly related species. The first successful somatic hybrid was formed in 1972 when Carlson and coworkers fused tobacco mesophyll protoplasts which demonstrated that this idea can be employed to enhance crops . Somatic hybridization is a technique involving the fusion of protoplasts (plant cells with their cell walls enzymatically removed) under lab conditions, contrasting sexual reproduction and genetic recombination through meiosis, and traditional breeding processes. The ability to combine nuclear and cytoplasmic genomes from different plant species (including those that cannot hybridize due to pre- or post-zygotic barrier) is unique to this process. Somatic hybrids obtained will have retained genetic material from both parent species, and may show new traits with improved qualities for agricultural, horticultural, or pharmaceutical purposes. Somatic hybridization has practical significance, as it can be used to introduce desirable traits such as disease resistance, stress tolerance and improved yield from one species into another, even when the two species are incompatible. This can be done, for example, by transferring disease resistance genes from their wild relatives to cultivated crops, thus improving their resistance to pathogens without sacrificing yield or quality. Somatic hybridization also facilitates the formation of cybrids (cytoplasmic hybrids) between the nucleus of one species and the cytoplasm of another, providing a means of transferring cytoplasmic characters such as male sterility or herbicide resistance.

Isolation of Protoplasts for Somatic Hybridization

The isolation of viable, fusion-competent protoplasts is the basis for somatic hybridization. The basic units for cell fusion are protoplasts, plant cells with their rigid cellulosic walls removed. Protoplast isolation



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techniques require careful optimization of several factors including enzyme concentrations that can digest the cell wall without harming the plasma membrane, osmotic conditions that can stabilize a cell lacking a cell wall, and meticulous-strategizing throughout the entire process to maintain cell viability as well as wall removal. This key process is involved in subsequent fusion events and subsequently determines the efficiency of hybrid plant regeneration. Choosing the suitable plant material is the first and probably the most important step in protoplast isolation. Sources may include different tissues such as leaves, stems, roots, callus cultures, cell suspensions and embryos. For instance, mesophyll cells in leaves are a popular choice because they are easy to isolate, are relatively consistent, and normally give high yields of protoplasts. The best tissue type can differ significantly between species, and cultivars within a species. Tobacco protoplasts, for example, can easily be isolated from leaf mesophyll, but protoplasts from cereals are frequently derived from embryogenic callus or cell suspensions because the differentiated tissues are more recalcitrant. The state of the donor tissue plays a significant role in protoplast yield and viability. Typically younger rapidly growing tissues produce more viable protoplasts than mature or senescent tissues. Plants grown in a controlled-environment (optimal light intensity, temperature and humidity) generally yield more consistent results

than field-grown materials. Dark adaptation of donor plants for a period of 24-48 hours prior to isolation can lead to improved protoplast yield by reducing starch accumulation in chloroplasts and increasing cell wall plasticity which enables better enzyme degradation. Another important factor in protoplast isolation is the enzymatic cocktail that is used for cell wall digestion. Plant cell wall consists of cellulose microfibrils within an amorphous matrix of hemicelluloses, pectins, and structural proteins, requiring the action of a suite of enzymes for their full degradation (Zhang et al., 2020). Cellulase is responsible for the breakdown of cellulose, the main structural component of primary walls, while pectinase digests the pectins present in the middle lamella



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in order to separate the cells. Depending on the characteristics of the cell wall of the target tissue, additional enzymes like hemicellulase, macerozyme, and driselase could also be used. Thus, a careful optimization of the concentration, purity, and ratio of these enzymes are needed for each plant species to ensure efficient wall degradation without cytotoxic effect. Protoplast stability is crucially dependent on the osmotic environment of enzymatic digestion and subsequent handling. Protoplasts have no cell walls to provide structural support and as such, are vulnerable to osmotic stress. Typically used in isolation and culture media at concentrations between 0.4 and 0.8 M, osmotic stabilizers act to help maintain cellular metabolism while preventing protoplast swelling and subsequent bursting, due to water influx through the membrane, by providing sufficient osmotic pressure (47, 48). The viscosity or density of osmoticum needs to be determined empirically for a particular species to prevent osmotic shock of the protoplast that can adversely affect cellular functions (Fujii et al., 2011; Monji et al., 2022).

Mechanism of Somatic Hybridization

Mechanism of Somatic Hybridization:

1. 1. Protoplast Isolation:

- Protoplasts are isolated from plant cells by enzymatic digestion of the cell wall using enzymes like cellulase and pectinase.
- This creates naked cells that can fuse with other protoplasts.
- Protoplasts can be isolated from various plant parts like leaves, roots, or fruits.

2. 2. Protoplast Fusion:

- Isolated protoplasts from different plant sources are brought into close contact.



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- Various methods are employed to induce fusion, including:
 - **Chemical methods:** Using agents like polyethylene glycol (PEG) or high pH solutions to destabilize the cell membranes and promote fusion.
 - **Electrofusion:** Applying electrical pulses to protoplasts, causing them to fuse.
- The fused protoplasts then form a hybrid cell containing genetic material from both parents.

3. 3. Hybrid Cell Selection:

- After fusion, a variety of techniques are used to identify and select the desired hybrid cells from the mixture.
- **Selection based on growth medium:** Hybrid cells may grow on a specific medium that is selective for the hybrid.
- **Visual selection:** Hybrid cells may be visually identified and separated based on differences in morphology or growth patterns.
- **Molecular markers:** Techniques like PCR or specific staining can be used to confirm the hybrid nature of cells.

4. 4. Plant Regeneration:

- Selected hybrid cells are cultured in a suitable nutrient medium to regenerate into whole plants.
- This involves inducing cell division, callus formation, and ultimately, the development of roots and shoots.
- The regenerated plants are then transferred to soil.



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Upon fusion, heterokaryons face a variety of fates that dictate the genetic makeup of hybrids. The nuclear fusion (karyogamy) can be spontaneous or requires stimulation by chemical treatments or culture conditions. This creates hybrid cells with a single nucleus but with chromosomes from both parents (at twice the normal number of chromosomes). On the contrary, one of the parental nuclei could slowly decay, while the other persists, incorporating even some fragments of genetic material from the decaying nucleus by fragmentation and assimilation. In certain instances, both nuclei exist without fusing, resulting in binucleate cells that can survive a number of division cycles before ultimately undergoing karyogamy or nuclear sorting. Somatic hybridization consequently has the potential to impart genetic effects additional to those observed for the nuclear genomes of hybrids. The cytoplasm contains the mitochondrial and chloroplast genomes which experience differing pathways of recombination and segregation as heteroplasmic cells divide. Organellar inheritance in somatic hybrids is also random but can also involve recombination between parental molecules, and preferred amplification of one maternal type, which is not usually the case during nuclear inheritance, which generally follows Mendelian principles. These processes create new cytoplasmic constituents that cannot be generated by sexual hybridization, including authentic recombinant organellar genomes with new gene arrangements.

Unit 4.3: Introduction to Fusion of Protoplasts



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Introduction to Protoplast Fusion

One of the major biotechnological breakthroughs in plant breeding and genetic engineering is protoplast fusion. Essentially, plant protoplasts cells without any rigid cell walls, containing only plasma membrane, cytoplasm and essential organelles are fused together to form hybrid cells, with genetic material from each parent. For this reason, this method overcomes the natural genomes inaccessibility which prevent hybridization among distantly related or crop incompatible plants species and will actively target the improve the crop traits in all ranges of alley cross-breeding methods. Protoplast fusion is a method that has been refined on the basis of concepts developed in the early 20th century, and significantly improved between the 1960s and 1970s. The successful creation of the first somatic hybrid plants by researchers like Peter Carlson proved that this method was indeed also practicable. These early successes triggered a flurry of advances in the discipline and shortly thereafter, protoplast fusion became a key tool in plant biotechnology. Protoplast fusion has numerous applications in agriculture and biotechnology. Through this method, cross species transfer of favorably useful characteristics, such as disease resistance, tolerance to stresses, and nutritional quality, is achieved. Transferring disease resistance genes from wild relatives into cultivated varieties has resulted in crops with improved pathogen resistance. Outside of agriculture, there are applications of protoplast fusion in cell biology and fundamental research, somatic hybridization and the synthesis of novel compounds by fusing cells with complementary biosynthetic pathways. Protoplast fusion is unique among genetic engineering techniques in that it can transfer whole genomes or large chromosomal sections, not single genes.

Mechanism of Protoplast Fusion

Protoplast fusion is a multifaceted biological process consisting of several major steps, starting from the break down of cell wall barrier into naked protoplasts, bringing protoplasts into proximity, fusion of



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the membranes of the protoplasts, and finally the integration of the genetic material from the two parental cells. For this reason it is essential to understand the mechanisms involved in each of these stages in order to optimize the efficiency and specificity of the protoplast fusion process. This takes place as the very first step in the fusion process, where protoplasts must be brought closer together. This proximity is required for subsequent membrane fusion events, as the plasma membranes of individual protoplasts need to overcome repulsive forces due to their negative surface charges. To promote this contact, many strategies have been developed, ranging from centrifugation, forcing protoplasts close proximity through gravitational forces, and the use of positively charged substances, such as poly-L lysine, which neutralizes the negative charge on protoplasts, to lower repulsion and turn adhesion. The fusion of plasma membranes occurs during a sequence of molecular rearrangements once the two cells are closely apposed. The transmembrane asymmetric lipid bilayer imbalance is one of the main factors leading to this process, resulting in localized membrane destabilization. Such destabilization may be induced by fusogenic agents that cause the disorder of phospholipid arrangement in the membrane. The membranes then become destabilized and temporary pores open at the points of contact, enabling the mixing of cytoplasmic contents.

Methods of Protoplast Fusion

Induction of protoplast fusion has been the subject of an evolving repertoire of methodologies since early demonstrations of the basic technique, and distinct approaches are now available, each of which has its own strengths, weaknesses, and applications. They can be categorized into chemical, physical, and electrical methods, but in modern studies more typically hybrid approaches that employ several of these strategies are used. Chemical fusion Continuous methods: Chemical fusion is the oldest and one of the most common methods to induce protoplast fusion. These techniques generally rely on the application of fusogenic agents that compromise membrane integrity,



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thereby facilitating fusion. The most frequently used chemical fusogen is polyethylene glycol (PEG) because of its effectiveness and relatively low toxicity. The fusion is mediated by PEG-dehydration force at the membrane surface and pulls protoplasts together, resulting in DNA driven fusion by the destabilization of membrane lipid motifs at areas of protoplast contact. Usually, calcium ions greatly treatment conditions (e.g. Ko et al., 2020; Li et al., 2018). Other chemical agents used for protoplast fusion besides PEG include sodium nitrate, which alters membrane permeability; dextran used in protoplast aggregation; and a number of lipophilic compounds that act directly on the membrane lipids to promote membrane adherence and fusion, which accelerates the process of fusion. While PEG-facilitated fusion is easy to perform and inexpensive, fusion can be inconsistent and is potentially harmful to certain protoplasts, with strict optimization of the PEG concentration, molecular weight, exposure time, and post-treatment conditions (e.g. Ko et al., 2020; Li et al., 2018). Other chemical agents used in protoplast fusion aside from PEG are sodium nitrate, which alters membrane permeability; dextran, used for protoplast aggregation; and some lipophilic substances that directly alter membrane lipids. Though less frequent than PEG because of a limitation in efficacy or specificity, these other chemical fusogens are utilized in specialized uses where PEG has lower efficiency or higher cytotoxicity. Protoplast fusion is a physical method that generally applies mechanical forces to closely associate protoplasts and promote membrane fusion through mechanical forces.

Factors Affecting Protoplast Fusion

Weighing biological, chemical, physical and methodological factors, the successful fusion of protoplasts and the regeneration of hybrid plants are the net result of a number of circumstantial factors. Knowledge of such elements and how they interact is essential for developing improved protocols for protoplast fusion, thereby increasing the efficiency and reproducibility of this methodology. The fusion success rate greatly depends on the physiological condition of



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the parent cells from which protoplasts are prepared. Protoplasts are generally more viable and female fusions competent from cells in the logarithmic phase of growth and not from stationary or senescent phase cells. This is in part because active growing cells generally have greater membrane fluidity and metabolic activity. Protoplast quality can also be affected by the age, health and growth conditions of the source tissue. For example, protoplasts obtained from young, etiolated tissues often have greater fusion capacity than those from mature, lignified tissues. In some cases, pre-treatments of source plants, like dark incubation or treatment with specific hormones, may improve protoplast quality and improve the subsequent efficiency of fusion. Another important factor determining fusion success is the quality of isolated protoplasts. Isolation and fusion procedures have to be done in such a way that protoplasts maintain their membrane integrity, metabolic activity and regeneration ability. The quality of protoplast is affected by the methods employed in the digestion of cell walls, the composition of enzyme mixtures, as well as the conditions (duration, temperature, pH) of enzymatic treatment. However, prolonged exposure to these enzymes may destroy the plasma membrane and intracellular structures, while inadequate treatment can leave some of the cell wall intact and subsequently hinder cell fusion. Moreover, osmotic stress experienced during isolation or later steps strongly impacts protoplast stabilization, with the application of hypotonic setups causing rupture and hypertonic environments causing overdehydration and metabolic trauma. In chemical fusion methods such as those with PEG the outcomes of fusions are notably determined by various facets.

4.3.1 Applications of protoplast fusion

In spite of which, it has developed into a tool of incredible utility with applications ranging across plant science, agriculture and biotechnology domains. This unique ability to break down barriers for traditional hybridization has led to many innovations in crop improvement, genetic analysis, and metabolic engineering. In crop



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improvement, protoplast fusion has been especially useful in transferring advantageous characteristics from wild relatives to cultivated species. In the case of somatic hybridization, for example disease resistance genes from wild *Solanum* species have been transferred to cultivated potato (*Solanum tuberosum*) leading to the development of varieties with increased resistance to late blight and other pathogens. Likewise, in the *Brassica* genus, the application of protoplast fusion has led to the creation of interspecific hybrids with the oil quality of *B. napus* and the disease resistance of *B. juncea*. And the list can continue: those examples show how the gene pool available for crop improvement is further expanded by protoplast fusion and underlines that breeders can make use of those valuable traits in sexually incompatible species. This process, known as protoplast fusion, has also been useful for generating new hybrid crops that express unique combinations of parental traits. As an example, the hybrid of radish (*Raphanus sativus*) and cabbage (*Brassica oleracea*) has intermediate leaf morphology and a special root structure.

4.3.2 A Brief Overview of Protoplast Fusion: Successes, Challenges, Opportunities, and Future Perspectives

Although in recent decades protoplast fusion technology has achieved noteworthy progress, several enduring issues are still limiting not only its broad application, but also its efficacy with different plant species. To meet these challenges, emerging applications will focus on providing new tools or utilizing new technology, which is a key frontier in the evolution of this field. Regeneration of whole, fertile plant from fusion products is one of the biggest technical challenges in protoplast fusion. Although detailed protocols for the isolation and fusion of protoplasts have been established for many species, the downstream processes of cell wall regeneration, prolonged cell division, embryogenesis or organogenesis, and plant development are often limiting steps, especially for monocotyledonous crops such as cereals



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and commercially important perennial species. The regenerative ability depends on multiple factors including, but not limited to, the molecular background of the fusion partners, protoplast physiological status, and applied culture conditions. Developing novel, desirable forms of plant regeneration demands deeper understandings of the molecular mechanisms involved in totipotency and morphogenesis and the design of regeneration protocols tailored to specific species or genotypes. Another major challenge is the genomic instability commonly found in somatic hybrids. Wide hybrids frequently display chromosome elimination, recombination, or rearrangements, which uncouple phenotypes from expected genotypes and reduce fertility. In terms of future possibilities, there are a number of emerging technologies and approaches that may well help overcome some of these challenges and broaden the utility of protoplast fusion. Genome editing tool (precise genome editing tool) is the answer to this challenges; the CRISPR-Cas systems integrated into the protoplast fusion system can be a useful technique to specifically modify the genome of the fusion products to improve stability, remove undesirable traits and/or to introduce precise genetic improvements. Advances in single-cell genomics and transcriptomics similarly present opportunities to characterize fusion products at unprecedented resolution, potentially allowing researchers to identify molecular signatures of successful hybrids and to select promising lines for further development. The use of microfluidic technologies in protoplast fusion provides an additional route for progression, facilitating more precise control over fusion parameters, increased throughput, and lower material requirements. These systems are capable of both basic research on the mechanisms of fusion and applied research on crop improvement. Moreover, advancing non invasive imaging approach to monitor of protoplast fusion and later development would provide a new avenue to shed light on the dynamics of these processes and reveal the crucial points for intervention or optimization. More than technical innovations, the rubric of protoplast fusion includes its extension to previously recalcitrant species, an important frontier. Conventional protoplast fusion methods were also



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difficult for many economically significant crops with respect to cereals, legumes and perennial species. Specifically tailored protocols that consider the unique physiological and developmental traits of these species could open more avenues to genetic improvement and trait transfer. These are exciting times for protoplast fusion, and we are seeing a resurgent interest in it with the possibility to move the field further forward, allowing us to witness its further potential as the future in plant science and crop improvement. By overcoming the ongoing technical, biological, and regulatory challenges through interdisciplinary strategies and novel technologies, protoplast fusion could go from a niche research technique for onesy-twozey entities to a ubiquitous part of agricultural innovation systems, enabling greener, higher yielding crops that will sustain the population of the future.

Summary

Somatic hybridization is a technique in plant biotechnology where protoplasts (plant cells without cell walls) from two different species or varieties are fused to create hybrid cells. Unlike sexual hybridization, which depends on natural crossing, somatic hybridization allows the combination of genetic material from distantly related species that cannot normally crossbreed. The process involves isolating protoplasts, inducing fusion using chemicals (like polyethylene glycol) or electrical methods, and regenerating the fused cells into whole plants through tissue culture. This method helps in transferring desirable traits such as disease resistance, stress tolerance, and improved nutritional quality. Somatic hybrids, also called **cybrids** (when only cytoplasmic transfer occurs), play an important role in crop improvement, overcoming incompatibility barriers, and broadening genetic diversity for plant breeding programs.

Somatic hybridization involves fusion of:

- A) Two gametes
- B) Two somatic cells



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- C) Two zygotes
- D) Two pollen grains

Answer: B) Two somatic cells

2. The primary goal of somatic hybridization is to:

- A) Produce seeds faster
- B) Combine desirable traits from two different plants
- C) Induce mutations in plants
- D) Enhance photosynthesis

Answer: B) Combine desirable traits from two different plants

3. Which technique is commonly used for protoplast fusion in somatic hybridization?

- A) Electroporation
- B) Agrobacterium-mediated transformation
- C) PEG (Polyethylene glycol)-mediated fusion
- D) CRISPR-Cas9

Answer: C) PEG (Polyethylene glycol)-mediated fusion

4. Protoplasts are:

- A) Cells with intact cell walls
- B) Cells without cell walls



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- C) Plant embryos
- D) Plant seeds

Answer: B) Cells without cell walls

5. Somatic hybridization can be used to:

- A) Transfer cytoplasmic traits
- B) Overcome sexual incompatibility
- C) Produce disease-resistant plants
- D) All of the above

Answer: D) All of the above

6. Which of the following is a limitation of somatic hybridization?

- A) Requires protoplast isolation
- B) Genetic instability of hybrids
- C) Technical complexity
- D) All of the above

Answer: D) All of the above

7. Cybrids are:

- A) Hybrids formed by sexual reproduction
- B) Cytoplasmic hybrids formed by somatic hybridization



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C) Hybrids formed by mutation

D) Hybrids between two fungi

Answer: B) Cytoplasmic hybrids formed by somatic hybridization

8. Which plant tissue is most commonly used for protoplast isolation?

A) Root tip

B) Leaf mesophyll

C) Stem bark

D) Seed coat

Answer: B) Leaf mesophyll

9. Somatic hybridization is especially useful in:

A) Animal breeding

B) Overcoming pre- and post-fertilization barriers in plants

C) Seedless fruit production

D) Enhancing photosynthetic efficiency

Answer: B) Overcoming pre- and post-fertilization barriers in plants

10. Which factor is essential for successful plant regeneration from fused protoplasts?

A) Plant growth regulators

B) High light intensity



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C) Low temperature

D) Mechanical stress

Answer: A) Plant growth regulators

Short answer type questions

- a. What is somatic hybridization in plant biotechnology?
2. Which type of cells are used in somatic hybridization?
3. Name two techniques used for protoplast fusion.
4. What is the main purpose of somatic hybridization in plants?
5. Define cybrid in the context of somatic hybridization.



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Unit 4.4: Cybrids

4.3.1 Introduction to Cybrids

Cybrids (Cytoplasmic hybrids) are an intriguing development in cellular biotechnology which is a result of a fusion between genetic engineering and cellular biology. They are uncommon cells, being formed by the merger of a cytoplasm (with mitochondria) of one organism with the nucleus of a different organism. Cybrids have nuclear genetic material from only one source like conventional hybrids but the cytoplasmic content in cybrids comes from a different cytoplasm donor; most often, the cytoplasm from another price of transported cell. This selective mixing generates a chimeric cell with particular properties that have provided immediate new directions for research in areas as diverse as evolutionary biology and medical therapeutics. Cybrids first came about in the 60s, when researchers started to experiment with techniques that fused different types of cells, but it was not until the 80s that the technology had matured to the point of being able to produce cybrids, that were consistently viable. These types of innovations arose as scientists already knew that mitochondria the organelles that provide energy to cells have a separate genome from the nuclear DNA. This mtDNA is maternally inherited and encodes proteins necessary for oxidative phosphorylation, energetic pathways in the cell. Through the manipulation of these unique genetic modules, scientists discovered they could engineer new cellular systems to investigate mitochondrial function, inheritance modes, and mechanisms of disease. Cybrids are situated at the crossroad of multiple biological disciplines, borrowing methods and concepts from cell biology, genetics, biochemistry and molecular biology. The important part is that it is a great model for looking at how mitochondrial genetics contributes to what a cell can do independently of what comes from the nuclear endogenous effects. The separation of these genetic material has been key to gaining insight into the interplay of nuclear and mitochondrial genomes in health and disease by studying how



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perturbations in this relationship might underlie pathological conditions in lower model organisms.

4.3.2 Mechanism of Cybrid Formation

The protocol for generating such cybrids is relatively complex and takes advantage of the intricate nature of cellular organization to allow for selective fusion of cytosolic constituents of one species with the nucleus of another. The process has improved considerably during this time, and modern methodologies yield better efficiency, specificity, and reproducibility. The mechanism by which cybrids form is also critical for understanding not only the scientific challenges associated with cybrid formation, but also the unique experimental and therapeutic potential of these experimental cellular constructs. Cybrids are formed mainly through the sequential elimination or impairment of certain cellular components from one individual, and then combination with the rest from the other individual. Different processing techniques, with strengths and weaknesses for specific research purposes, have been established in order to obtain this. Cybrids have been predominantly created using the most common method that comprises enucleation or the enucleated donor cell which retains a considerable amount of cytoplasmic material including mitochondria. A range of methods may be used for enucleation, including centrifugation to take advantage of differences in density between the nucleus and cytoplasm to physically separate these components. Another method utilizes micromanipulators to surgically extract the nucleus through microdissection techniques. In more recent strategies, cytochalasin B is used to destroy actin filaments and help mechanically extrude the nucleus outwards, before forming cytoplasts (cellular fragments containing cytoplasm and organelles but without a nucleus). At the same time, the recipient cells that will supply the nuclear DNA are prepared in a different way. Such cells are often treated to strip them of their original mitochondria or to make them nonfunctional. This can be accomplished through common practices like treatments with rhodamine 6G or with ethidium bromide, both of which interfere with mitochondrial DNA replication and transcription,



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ultimately resulting in the generation of mitochondrion-free (rho-zero or ρ^0) cells. Because these mitochondria-depleted cells lack endogenous mitochondrial function, they are ideal for the transfer of foreign mitochondria, allowing the researchers to isolate and study the effects of the transplanted mitochondria. After the cytoplasm (which carries the donor mitochondria) and the recipient cell (source of the nucleus) are prepared, they are fused to generate the cybrid. Cell fusion is traditionally mediated by polyethylene glycol (PEG), which changes membrane fluidity to encourage membrane fusion, or through electrofusion, which uses short electrical pulses to destabilize cell membranes temporarily and make them fuse. Recently, viral fusogenic proteins have been employed, improving the specificity and efficiency of the fusion reaction. After fusion, successful cybrids are selected from the hybrid cells formed. This combination usually takes advantage of the synergistic weaknesses following the preparatory phases. For example, if the recipient cells are ρ^0 cells (defective in their mitochondrial function) and cannot grow in media devoid of uridine and pyruvate (since these compounds can bypass a functional mitochondrion), then cybrids that acquire functional mitochondria from the cytoplasm will grow in this selective medium. Conversely, if there are nuclear genes required for cytoplasm longevity, only those cells that having incorporated a functional version of their entire complement of nuclear DNA will survive. A second approach to cybrid formation is to directly transfer isolated mitochondria into recipients. This approach circumvents the requirement for total cellular fusion by concentrating on targeted importation of mitochondria. Methods for enrichment and introduction of isolated mitochondria into recipient cells include, but are not limited to, co-incubation under specific conditions to enhance uptake, precise delivery through microinjection, and use of cell-penetrating peptides to allow targeted mitochondria entry into recipient cells.

Applications of Cybrids



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Cybrids represent a versatile mode with applications ranging from basic science to medicine, agriculture: conservation biology, and biotechnology. Their chimeric nature (both a single donor's nuclear DNA and another's mitochondrial DNA) opens a rare window for scientists studying cellular biology, therapeutic strategies, and evolutionary questions. The applications of cybrid technology are unlimited in number and unproblematic in nature, as cybrid tech improves society by solving previously unresolvable problems across fields. Cybrids are extremely helpful in providing a model to better characterize the integrated contribution of nuclear and mitochondrial genomes, which is essential for basic biological research. The mitochondria, now an organelle of integral function and significance to the cell, were once a standalone prokaryote that took part in endosymbiosis with eukaryotic ancestors almost two billion years ago. This evolutionary history has led to a distinct genetic configuration in which mitochondrial function is reliant on proteins encoded by both mitochondrial DNA (mtDNA) and nuclear DNA. Cybrids provide researchers with a systematic way to explore this relationship by generating cells with unique combinations of nuclear and mitochondrial genomes, unearthing how these two genetic systems co-evolved and how they continue to interact in contemporary cells. Immediately following this development, cybrids found perhaps their most significant utility in the investigation of mitochondrial diseases — heterogeneous disorders that result from mutations in the mitochondrial DNA affecting about 1 in 5,000 overall. These diseases are often neurological, muscular, or metabolic in nature and have been difficult to study due to the peculiar genetics of mitochondria, including maternal inheritance, heteroplasmy (the presence of both mutant and wild-type mtDNA in the same cell), and threshold effects (symptoms only appearing once the proportion of mutant mtDNA has surpassed a threshold). Cybrids offer a defined experimental system through which investigators can study how specific mutations in mitochondrial genetics drive cellular function within the context of a specific nuclear genetic background, isolating the effects of mitochondrial genetic



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variation on disease progression. By generating cybrids containing varying amounts of mutant mtDNA, threshold effects can be studied in an attempt to determine the minimum amount of mutant mitochondria necessary to affect cellular function. This is critical information to have for an understanding of disease progression and the development of potential interventions. Additionally, by comparing cybrids possessing the same mitochondrial mutations but different nuclear backgrounds, researchers can discover nuclear genetic factors that may alter the expression of mitochondrial disorders, effectively finding potential new therapeutic targets. In addition to their use in basic research, cybrids have direct applications for developing therapies for mitochondrial diseases. Mitochondrial replacement therapy (MRT) is one of the most hopeful and contentious applications, and involves creating embryos with nuclear DNA from the intended parents, but mitochondria from a donor with health mtDNA.

Summary

Protoplast culture is a plant tissue culture technique that involves the isolation and cultivation of protoplasts—plant cells that have had their cell walls enzymatically removed. This method enables direct access to the plasma membrane and internal cell components, making it valuable for various experimental and biotechnological applications. Protoplasts can be regenerated into whole plants under controlled conditions, allowing scientists to study cellular processes, perform genetic modifications, and create somatic hybrids through protoplast fusion. This technique plays a crucial role in plant research, genetic engineering, and the development of improved crop varieties.

Which enzyme is commonly used for isolating protoplasts from plant tissues?

a) Cellulase



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- b) Protease
- c) Amylase
- d) Lipase

3. What is the purpose of protoplast fusion?

- a) To regenerate whole plants
- b) To create hybrids between different species
- c) To produce secondary metabolites
- d) To induce genetic mutations

4. Which of the following techniques involves the fusion of protoplasts?

- a) Somatic embryogenesis
- b) Protoplast fusion
- c) Micropropagation
- d) Cloning

5. What are cybrids?

- a) Hybrid cells with two nuclei
- b) Cells with cytoplasmic and nuclear fusion
- c) Hybrid plants with identical traits
- d) Somatic embryos

6. What is the benefit of using protoplast fusion in crop improvement?

- a) Increased resistance to diseases
- b) Faster growth rates
- c) Hybrid vigor and genetic diversity



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d) Reduced cost of production

7. What is the most common source of protoplasts in plant tissue culture?

- a) Roots
- b) Leaves
- c) Meristematic tissue
- d) Anthers

8. In somatic hybridization, which part of the protoplasts from different species is combined?

- a) Nucleus
- b) Cytoplasm
- c) Mitochondria
- d) Chloroplast

9. What is a major factor that influences the success of protoplast fusion?

- a) Protoplast size
- b) The fusion medium
- c) Temperature
- d) Both b and c

10. What is the main application of cybrids in plant breeding?

- a) Disease resistance
- b) Genetic modification
- c) Hybrid vigor
- d) Fertility restoration



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Short Questions:

1. Define protoplast culture.
2. What are the main steps involved in protoplast isolation?
3. How does protoplast fusion contribute to the development of hybrid plants?
4. What are the advantages of somatic hybridization in plant breeding?
5. Describe the process of protoplast fusion.
6. How does the fusion medium affect protoplast fusion?
7. What is the role of cybrids in overcoming sterility in hybrids?
8. Explain the significance of protoplasts in genetic research.
9. What factors influence the viability of protoplasts in culture?
10. Discuss the applications of cybrids in crop improvement.

1. Explain the process of protoplast isolation and its significance in plant tissue culture.

Discuss the mechanism and applications of somatic hybridization in plant breeding.

3. How does protoplast fusion contribute to creating new plant varieties?
4. Describe the formation and applications of cybrids in agriculture.
5. Explain the methods used for protoplast fusion and their limitations.
6. How can protoplast fusion be used for improving crop resistance to diseases?
7. What is the significance of using cybrids in overcoming incompatibility



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barriers between species?

8. Discuss the factors that affect the success of protoplast fusion.

9. Describe the role of protoplast culture in plant biotechnology.

10. What are the practical applications of protoplast fusion and somatic hybridization in modern agriculture?

MODULE 5



INTRODUCTION TO THE PRODUCTION OF TRANSGENIC PLANTS

Objective:

To explore the various techniques used to produce transgenic plants, the methods of transformation, and the applications of plant tissue culture in genetic engineering.

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Unit 5.1: Transgenic Plants

5.1.1 Introduction to Transgenic Plants

Genetically modified (GM) plants are one of the most important technological achievements of both modern agriculture and plant biotechnology. These “non genetically modified” plants contain genes that have been manipulated by genetic engineering methods, not by traditional breeding methods. These genes are usually either taken from other plant species, bacteria, viruses, fungi, and animals or synthetically made in labs. Creating transgenic plants primarily aims to incorporate new characteristics that can improve the value of the plants for agricultural and nutritional purposes, increase resistance against pests and diseases, enhance tolerance to environmental stress, or spur their use for pharmaceutical purposes. Transgenic plants were first conceived in the early 1980’s, and the first successful plant genetic transformation was described in 1983. With the successful use of *Agrobacterium tumefaciens* as a vector for transferring foreign DNA into tobacco plants, this technological breakthrough was accomplished. However, great strides have been made since then and many transgenic crop varieties are now commercially cultivated globally. Among the most common transgenic crops are soybeans, corn, cotton, and canola, which have been developed mainly for herbicide tolerance and insect resistance. By incorporating traits such as insect resistance, farmers can minimize their reliance on chemical pesticides, reducing environmental pollution and production costs. Likewise, herbicide-tolerant crops enable better weed control, which again leads to reduced mechanical tillage of soil and thus less soil erosion. Although transgenic plants have the potential to help solve many problems, they have spent the past several years in the scientific spotlight and under heavy public debate. Stocking fish is widely regarded as a biodegradable underclass of food and the willingness to eat it flows from one regulation or lack thereof which could be social or legal and its consequences can range from environmental to economic to even safety concerns about their consumption. The traditional regulatory frameworks evaluate the risks



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of transgenic plants, including allergenicity, toxicity, gene flow to wild relatives and impact on non-target organisms. Transgenic plant technology by using genetic engineering techniques is a promising area for food. And today, plant research includes the creation of plants with improved nutritional content (biofortification), drought and salinity tolerance, optimised photosynthesis, pharmaceuticals or industrial compounds. The domain also overlaps with emerging technologies like genome editing, which enables more precise forms of genetic modification.

5.1.2 Techniques for Generating Transgenic Plants

grobacterium-mediated gene transfer:

- This method utilizes the bacterium *Agrobacterium tumefaciens*, which naturally transfers a portion of its DNA (T-DNA) into plant cells.
- Scientists modify the bacterium's Ti plasmid by replacing the disease-causing genes with the desired gene of interest.
- The modified *Agrobacterium* then infects plant cells, and the T-DNA, carrying the new gene, integrates into the plant's genome.
- This approach is widely used, particularly for dicotyledonous plants.

2. Particle bombardment (Gene Gun):

- Also known as biolistics, this method involves coating microscopic particles (gold or tungsten) with DNA and then shooting them into plant cells using a high-velocity device.
- The DNA-coated particles penetrate the cell walls and membranes, and the DNA can integrate into the plant's genome.
- This technique is versatile and can be used for a wide range of plant species, including monocots like wheat and rice.



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3. Electroporation:

- This method uses short electrical pulses to create temporary pores in the cell membrane, allowing DNA to enter the cell.
- The plant cells are mixed with the DNA in a solution, and then exposed to an electrical field.
- This technique is particularly useful for protoplasts (plant cells without cell walls).

4. Microinjection:

- This is a direct method where DNA is physically injected into the plant cell's nucleus using a fine needle.
- It requires specialized equipment and expertise, but it can be highly efficient for certain cell types.

5. Other methods:

- **Chemical-mediated gene transfer:**

Techniques like PEG-mediated transfer utilize chemicals like polyethylene glycol to facilitate DNA entry into cells.

- **Virus-mediated gene transfer:**

Plant viruses can be engineered to carry foreign genes and deliver them into plant cells during infection.

Example

Agrobacterium tumefaciens is a bacterium commonly used to create transgenic plants. It naturally transfers a segment of its DNA (T-DNA) into plant cells, inducing tumor formation. Scientists exploit this ability by replacing the tumor-inducing genes on the T-DNA with desired foreign genes, allowing for the genetic modification of plants. This method, known as *Agrobacterium*-mediated transformation, is a widely used and effective technique for generating transgenic plants.

1. *Agrobacterium*'s Natural Role:



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- Agrobacterium tumefaciens is a soil bacterium that causes crown gall disease in plants.
- It infects plants through wounds, typically on roots or stems.
- It transfers a specific DNA segment (T-DNA) from its Ti plasmid into the plant's genome.
- The T-DNA contains genes that, when expressed, cause uncontrolled cell growth, leading to tumor formation (crown galls).

2. Transformation Process:

- Scientists utilize this natural gene transfer mechanism to introduce new genes into plants.
- They replace the tumor-inducing genes on the T-DNA with the desired foreign gene(s).
- The modified Agrobacterium is then used to infect plant cells, typically through co-cultivation.
- The T-DNA, containing the foreign gene, integrates into the plant's genome.

3. Key Components and Mechanisms:

- **Ti Plasmid:**

The large, circular DNA molecule in Agrobacterium that carries the T-DNA and other genes involved in the transfer process.

- **T-DNA:**

The specific DNA segment that is transferred into the plant cell and integrates into its genome.

- **Virulence Genes (vir genes):**

Genes on the Ti plasmid that are essential for the transfer of T-DNA.

- **Type IV Secretion System (T4SS):**



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The bacterial machinery that delivers the T-DNA and other proteins into the plant cell.

4. Advantages of Agrobacterium-mediated Transformation:

- **Efficiency:** It is a relatively efficient and reliable method for introducing foreign genes into plant cells.
- **Cost-effectiveness:** Compared to other transformation methods, it is generally more economical.
- **Reproducibility:** The process is highly reproducible, allowing for consistent results.
- **Large DNA Fragments:** It can accommodate the transfer of relatively large DNA fragments.
- **Targeted Integration:** In some cases, it can be used for targeted integration of genes into specific locations in the plant genome.

5. Applications:

- Generating transgenic plants for research purposes, allowing scientists to study gene function and plant development.
- Developing crops with improved traits, such as disease resistance, pest resistance, herbicide tolerance, and enhanced nutritional value.
- Producing pharmaceuticals and other valuable compounds in plants.

6. Limitations:

- Not all plant species are susceptible to Agrobacterium infection.
- Occasionally, the transformation process can lead to unintended mutations or rearrangements in the plant genome.



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- The efficiency of transformation can vary depending on the plant species and the specific genes being introduced.

Protoplast Transformation

protoplast is a plant cell that has its cell wall removed, usually through enzymatic digestion. Once the cell wall is removed, different techniques are available to introduce DNA into protoplasts such as PEG treatment, electroporation or microinjection. In this mode of transformation, the protoplasts are subjected to treatment with PEG along with DNA. The PEG changes the membrane permeability, so that the DNA can get through the cell. In electroporation, a short electrical pulse is applied to protoplasts, leading to the establishment of temporary pores in the membrane with DNA entering through these pores. Microinjection, which is not as widely done because it is very labor-intensive, consists in sending the DNA directly into the protoplast by means of a fine glass needle. The protoplasts are cultured in a medium after transformation, which allows for the regeneration of cell walls and cell division forming callus tissue that can regenerate into whole plants. Methods of protoplast transformation Universal in general for can be done ligne of plants But, they need specific skills and equipment, while regeneration of entire plants from protoplast is often problematic for many species.

A New Strategy: Gene Editing

The third-generation genome editing tools, such as CRISPR-Cas9, TALEN, and ZFN, will provide more accurate genetic modification for plants. Utilizing these technologies, it is possible to make targeted alterations in specific sites in the genome that includes, gene knockout, gene replacement, or targeted alterations in the DNA sequence. Most genetically edited plants are generated by first transforming them with the editing components (e.g., the Cas9 protein and guide RNA molecule for CRISPR) using techniques that resemble those used to introduce the classical



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transgene. The big difference, though, is that once the desired genetic change has occurred in the plant, the editing machinery can be segregated out in later generations, resulting in plants that potentially do not contain any foreign DNA. This has also raised the question of whether genome-edited plants should be subject to the same regulations as transgenic plants, if in fact they lack foreign DNA. Genome-edited plants which have been modified in a way that mimics traditional breeding are currently covered by different regulations in different countries.

Selection and Regeneration of Transgenic Plants

Selection and regeneration are two critical steps in the development of transgenic plants. It is often a requirement to identify and isolate the plant cells that have undergone transformation with little or no background noise and then to regenerative they back to whole plants. In this segment, the techniques and challenges related to such processes are discussed.

Isolation of Transformed Cells

A vast majority of cells that undergo transformation processes fail to insert the foreign DNA into their genome. Thus, robust selection systems are needed to segregate these cells from a vast background of non-transformed counterparts.

Selectable Marker Genes

One of the most widely-adopted selection methods utilizes selectable marker genes that provide a specific trait which allows transformed cells to survive whereas non transformed cells are susceptible to the same condition that would hamper their growth. Selectable markers most commonly used are:

Antibiotic Resistance Genes

Examples include nptII (neomycin phosphotransferase II) which is responsible for resistance to kanamycin and related antibiotics or hpt



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(hygromycin phosphotransferase) which is responsible for resistance to hygromycin. Every transformed cell that carries the resistance gene will survive and proliferate on the media containing respective antibiotic.

Herbicide resistance genes

This category includes the bar gene (phosphinothricin acetyltransferase), which provides resistance to phosphinothricin (glufosinate), and EPSPS (5-enolpyruvylshikimate-3-phosphate synthase), providing glyphosate resistance, among many others. As with antibiotic selection, only those cells expressing these genes will grow on media containing the relevant herbicide.

Marker-Free Strategies

The presence of selectable marker genes in transgenic plants is a cause for concern, specifically for those that confer antibiotic resistance, as many higher plants currently grown are of commercial importance. These concerns include possible horizontal gene transfer to other organisms and the allergenic or toxic potential of the marker proteins. To alleviate these concerns, several approaches have been developed for the generation of marker-free transgenic plants:

Co-transformation and Segregation:

The gene of interest and the selectable marker are provided in different DNA molecules. Conventional breeding chooses plants, allows sexual reproduction, and selects descendants that still have the desired gene but not the marker gene (thanks to independent recombination).

Site-Specific Recombination

Marker gene excision (e.g., Cre/lox, FLP/ FRT) following selection includes the removal of the marker altogether. Recombinase gene (Cre or FLP) is conditioned either by crossing with plant carrying the recombinase expression or by inducible expression.

Transposons-Based Systems



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Its marker gene is flanked by transposon sequences, which may allow its further removal by transposition.

Positive-Negative Selection:

A two-step selection process. By using a positive selection, the transformed cells are selected first. Then a negative selection during regeneration (in the case of plants) or over several generations (where selection is for the progeny) is carried down to select for cells that lose the marker.

Organogenesis:

Shoot buds and roots are formed directly from callus tissue or explants. This is usually done by adjusting the ratio of plant growth regulators (auxins and cytokinins primarily) in the culture medium. This shows that when there is a high cytokin to auxin ratio, it generally promotes the formation of shoots while a high auxin to cytokin ratio will lead to the formation of roots. Direct organogenesis (buds develop directly from the explant) and indirect organogenesis (buds develop from an intervening callus phase) are available.

Somatic Embryogenesis:

Referred to the organogenesis, it can be defined as a process of embryo formation from somatic (non-reproductive) cells and the conversion of these cells into whole plants. Somatic embryogenesis, embryos undergo similar developmental stages to zygotic embryos but are not fertilized. This process is typically induced by high levels of auxin, after which auxin is removed. Somatic embryogenesis can be either direct or indirect. The pathway of regeneration may be mutually exclusive, and therefore, the choice in regeneration pathway depends on the plant species, the type of explant used and the goals of the transformation project. As species respond better to one pathway than another, there may be also better compatible transformation methods with specific regeneration methods.



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Summary

Transgenic plants are genetically modified plants that carry foreign genes inserted into their genome, enabling them to exhibit desirable traits such as pest resistance, herbicide tolerance, improved nutritional value, or stress tolerance. The production of transgenic plants involves biotechnology techniques, primarily gene transfer methods such as *Agrobacterium*-mediated transformation and gene gun (biolistic) method.

MCQs: Introduction to the Production of Transgenic Plants

1. What is a transgenic plant?

- A) A plant that has been cloned from tissue culture
- B) A plant that contains genes from another species
- C) A plant grown without soil
- D) A plant that produces only male flowers

Answer: B) A plant that contains genes from another species

2. Which of the following is commonly used as a vector for gene transfer in plants?

- A) *Escherichia coli*
- B) *Bacillus thuringiensis*
- C) *Agrobacterium tumefaciens*
- D) *Saccharomyces cerevisiae*



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Answer: C) *Agrobacterium tumefaciens*

3. The Ti plasmid in *Agrobacterium tumefaciens* is important because it:

- A) Helps in photosynthesis
- B) Transfers genes into the plant genome
- C) Produces antibiotics
- D) Enhances plant respiration

Answer: B) Transfers genes into the plant genome

4. Which method is widely used for direct gene transfer into plant cells without *Agrobacterium*?

- A) Gene gun (biolistic method)
- B) CRISPR-Cas9
- C) PCR
- D) RNAi

Answer: A) Gene gun (biolistic method)

5. Marker genes in transgenic plant production are used to:

- A) Identify successfully transformed cells
- B) Promote flowering
- C) Increase yield
- D) Reduce plant height



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Answer: A) Identify successfully transformed cells

6. Which plant tissue is commonly used for transformation in tissue culture?

- A) Leaves
- B) Meristematic tissues
- C) Root hairs
- D) Seed coat

Answer: B) Meristematic tissues

7. What is the primary purpose of producing transgenic plants?

- A) To study photosynthesis only
- B) To develop plants with desired traits like pest resistance or herbicide tolerance
- C) To replace traditional farming completely
- D) To make plants edible for humans

Answer: B) To develop plants with desired traits like pest resistance or herbicide tolerance

8. Which of the following is an example of a commercially important transgenic crop?

- A) Golden rice



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- B) Spinach
- C) Wheatgrass
- D) Mustard

Answer: A) Golden rice

9. The process of integrating foreign genes into the plant genome is called:

- A) Gene silencing
- B) Gene cloning
- C) Genetic transformation
- D) Genetic crossing

Answer: C) Genetic transformation

10. Which technique is used to confirm the presence of a transgene in a plant?

- A) PCR (Polymerase Chain Reaction)
- B) Gel electrophoresis of plant sap
- C) Soil testing
- D) Leaf staining

Answer: A) PCR (Polymerase Chain Reaction)

The process generally includes:



Short answer type question

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1. What is a transgenic plant?
2. Name the commonly used methods for producing transgenic plants.
3. What is the role of *Agrobacterium tumefaciens* in plant genetic engineering?
4. Define gene cloning in the context of plant transformation.
5. What is a selectable marker gene, and why is it important?

Unit 5.2: Techniques of Transformation

Transgenic plants are created by introducing foreign DNA into a plant's genome, and this can be achieved through various techniques. Key methods include *Agrobacterium*-mediated transformation, particle bombardment (gene gun), electroporation, microinjection, and direct DNA uptake methods like PEG-mediated transformation.

***Agrobacterium*-mediated transformation:** This process uses the bacterium *Agrobacterium tumefaciens*, which has a natural tendency to insert some of its DNA into plant cells. Scientists alter the DNA of the bacterium to incorporate the gene to be inserted and then transferred into the plant genome. It is widely used because it is efficient and not very costly.

2. Particle bombardment (Gene Gun): This technique involves coating microscopic gold or tungsten particles with DNA containing the desired gene and then firing these particles into plant cells or tissues using a



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gene gun. The DNA is then incorporated into the plant's genome. This method is useful for a wide range of plant species.

3. Electroporation: In this method, a high-voltage electrical pulse is applied to a mixture of plant protoplasts (cells without cell walls) and DNA, creating temporary pores in the cell membrane through which the DNA can enter. The protoplasts are then regenerated into whole plants.

4. Microinjection: This technique involves directly injecting the desired DNA into plant cells, typically the nucleus, using a fine glass needle or micropipette. It's a more precise method but can be technically challenging.

These techniques are crucial for introducing desirable traits into plants, such as disease resistance, herbicide tolerance, or improved nutritional content. Tissue culture techniques are often used in conjunction with these methods to regenerate whole plants from transformed cells.

5.2.1 Chemical and Physical Transformation

Chemical and physical transformation methods represent important alternatives to biological approaches, particularly for plant species that are recalcitrant to *Agrobacterium*-mediated transformation. These methods rely on various physical or chemical forces to breach the plant cell wall and membrane barriers, allowing direct introduction of DNA into plant cells. Unlike biological methods, these approaches do not depend on the natural DNA transfer machinery of microorganisms.

Polyethylene Glycol (PEG)-Mediated Transformation

PEG-mediated transformation is a chemical method widely used for transforming protoplasts (plant cells with cell walls removed). The technique involves treating protoplasts with a solution containing DNA and polyethylene glycol, which alters membrane permeability and facilitates DNA uptake. The process begins with the isolation of protoplasts using enzymatic digestion of plant cell walls. The protoplasts are then suspended in a solution containing the DNA to be



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transferred, along with PEG and divalent cations such as calcium, which facilitate DNA binding to the cell membrane. The PEG causes the protoplasts to clump together and destabilizes the plasma membrane, creating temporary pores through which DNA can enter the cell. Following DNA uptake, the protoplasts are washed to remove PEG and placed in a culture medium for regeneration. PEG-mediated transformation offers several advantages, including its simplicity, low cost, and ability to transform multiple plant species. It is particularly useful for transient expression studies and for species where other methods yield poor results. However, the technique has limitations, including the technical challenge of isolating viable protoplasts, the difficulty of regenerating whole plants from protoplasts for many species, and relatively low transformation efficiencies compared to some other methods. Notable applications of PEG-mediated transformation include functional genomics studies in model plant systems like *Arabidopsis* and tobacco, transient gene expression analyses, and transformation of organelles such as chloroplasts, which is difficult to achieve with *Agrobacterium* mediated methods.

Electroporation

Electroporation is a physical method that uses brief electrical pulses to create temporary pores in the cell membrane, allowing DNA to enter the cell. This technique can be applied to various plant materials including protoplasts, intact cells, tissues, and even seeds. The electroporation process involves placing plant cells or tissues in a buffer solution containing the DNA to be transferred. The mixture is subjected to short electrical pulses of high voltage, which temporarily destabilize the cell membrane creating microscopic pores. DNA molecules in the surrounding medium can then move into the cell through these pores. Following the electrical pulse, the membrane reseals, trapping the DNA inside the cell. The efficiency of electroporation depends on several parameters including the strength and duration of the electrical field, the ionic composition of the electroporation buffer, temperature, DNA concentration, and the physiological state of the target cells. These



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parameters must be optimized for each plant species and tissue type. Electroporation offers advantages such as simplicity, versatility across different plant materials, and the ability to introduce DNA without vector constraints, making it suitable for delivering large DNA fragments. However, it also has limitations, including potential cell damage from electrical shock, variable transformation efficiencies, and the challenge of regenerating whole plants from electroporated cells, particularly for monocots. Electroporation has been successfully applied in transforming cereals like rice, maize, and wheat, where it serves as an important alternative to *Agrobacterium*-mediated methods. It is also widely used for transient expression studies and for studying protein localization and function in plant cells.

Microinjection

Microinjection is a direct physical method for DNA delivery that involves the mechanical injection of DNA directly into plant cells, typically into the nucleus or cytoplasm, using a fine glass micropipette. This highly precise technique allows for controlled delivery of DNA to specific cellular compartments. The microinjection process requires specialized equipment including a micromanipulator for precise control of the micropipette, a microscope for visualization of the target cell, and a microinjector for controlled delivery of the DNA solution. The target cells must be immobilized, and the DNA solution is drawn into a micropipette with a tip diameter of 0.5-1.0 μm . The micropipette is then inserted through the cell wall and membrane, and a precise volume of DNA solution is injected directly into the cell. Microinjection offers unique advantages, including the ability to deliver DNA to specific cellular compartments, control over the amount of DNA delivered, and the potential to process and transfer of T-DNA from the bacterium to the plant cell. In nature, the T-DNA contains genes that encode enzymes for the synthesis of plant hormones (auxins and cytokinins) and opines (amino acid derivatives). These genes, once expressed in transformed plant cells, cause uncontrolled cell proliferation (tumor formation) and the production of opines, which serve as a carbon and



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nitrogen source specifically for *Agrobacterium*. The T-DNA is defined by 25-base-pair border sequences (right and left borders) that flank the genes to be transferred. The VirD1 and VirD2 proteins recognize these border sequences and create a single-stranded nick in the bottom strand of the T DNA. VirD2 then covalently attaches to the 5' end of the single-stranded T-DNA (T strand) and guides it through the bacterial cell membrane. The T-strand is coated with VirE2 proteins, forming a T-complex that protects it from nuclease degradation during transfer. The T-complex is transferred into the plant cell through a Type IV secretion system formed by VirB proteins. Once inside the plant cell, nuclear localization signals on VirD2 and VirE2 guide the T-complex to the nucleus. The T-DNA then integrates into the plant genome through a process that likely involves the plant's DNA repair mechanisms. This natural process has been harnessed for plant transformation by replacing the tumor-inducing genes on the T-DNA with genes of interest, while retaining the essential border sequences required for DNA transfer. transform specific cells within a tissue. It is particularly valuable for studying large cells, such as embryo cells, and for applications requiring precise spatial control of transformation.

Binary Vector Systems

Modern *Agrobacterium*-mediated transformation typically employs binary vector

systems, which represent a significant improvement over earlier systems that used

modified Ti plasmids. Binary systems separate the vir genes and the T-DNA onto two

different plasmids, making the construction and manipulation of transformation vectors

much more manageable.

In a binary vector system:



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1. The disarmed Ti plasmid (helper plasmid) contains the vir genes needed for T-DNA processing and transfer but lacks the T-DNA region. This plasmid is maintained in the Agrobacterium strain.

2. The binary vector is a smaller plasmid that contains the engineered T-DNA region with the genes of interest flanked by the right and left border sequences. This plasmid can replicate in both *E. coli* (facilitating molecular cloning) and Agrobacterium. When both plasmids are present in the same Agrobacterium cell, the vir proteins from the helper plasmid can act in Trans to transfer the T-DNA from the binary vector into the plant cell.

Binary vectors typically include several key components:

- Multiple cloning sites within the T-DNA region for convenient insertion of genes of interest
- Selectable marker genes (e.g., antibiotic or herbicide resistance) for identifying transformed plants
- Reporter genes (e.g., GUS, GFP) for visualizing transformation events
- Appropriate promoters and terminators for efficient expression of introduced genes.
- Origins of replication for maintenance in both *E. coli* and Agrobacterium
- Bacterial selectable markers for maintaining the plasmid in bacterial hosts.

Numerous binary vectors have been developed with variations in these components to suit different transformation needs. Modern vectors often include gateway recombination sites for facilitating cloning, multiple restriction sites for flexible gene insertion, and various promoters for controlling gene expression in different plant tissues or developmental stages. The development of binary vector systems has greatly facilitated plant transformation by simplifying vector



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construction and improving transformation efficiencies across a wide range of plant species.

Floral Dip Transformation

The floral dip method, primarily used for *Arabidopsis thaliana*, is remarkably simple

and avoids tissue culture steps. The procedure involves:

1. Growing plants until they produce inflorescences
2. Immersing the developing floral tissues in an *Agrobacterium* suspension
containing surfactant
3. Allowing plants to mature and set seeds
4. Selecting transformed seedlings from the harvested seeds using appropriate

selection markers

Agrobacterium targets the female gametophyte, resulting in transformed seeds. While extremely convenient for *Arabidopsis*, this method has limited applicability to other plant species, though modified versions have been developed for some related species.

In Plant Transformation

In planta transformation methods target various plant tissues in intact plants, avoiding

extensive tissue culture. Besides the floral dip, other in planta approaches include:

Seed imbibition:

Seeds are imbibed in *Agrobacterium* suspension, allowing bacteria to infect the developing embryo

Pollen transformation:



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Pollen is exposed to *Agrobacterium* before pollination.

Infiltration of mature tissues

: *Agrobacterium* is infiltrated into leaves or other tissues using vacuum or pressure. These methods offer the advantage of simplicity and are particularly valuable for species recalcitrant to tissue culture. However, they typically yield lower transformation efficiencies than methods involving tissue culture.

Explant Transformation

Beyond leaf discs, various plant explants can be used for *Agrobacterium*-mediated transformation, including:

Cotyledons:

Young cotyledons from germinated seedlings

Hypocotyls

: The stem below the cotyledons in young seedlings

.

Stem segments

: Internodal sections from stems

Root segments:

Sections of young roots

.

Embryogenic callus:

Undifferentiated tissue with embryogenic potential

.

Somatic embryos

: Embryos developed from somatic tissues



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The choice of explant depends on the plant species, the regeneration capacity of

different tissues, and the accessibility of tissues to *Agrobacterium* infection.

Co-cultivation Conditions

Regardless of the specific methodology, several factors must be optimized for successful

Agrobacterium-mediated transformation:

Factors Affecting Transformation Efficiency

Numerous factors influence the efficiency of *Agrobacterium*-mediated transformation,

and optimization of these factors is critical for successful transformation of different

plant species:

Plant Factors

Genotype

: Different cultivars or varieties of the same species often show variable susceptibility to *Agrobacterium* infection

Tissue type and age

: Younger, actively dividing tissues are generally more amenable to transformation

Physiological state:

Plants grown under optimal conditions typically show better transformation

Endogenous hormone levels:



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These influence both susceptibility to infection and regeneration capacity.

Bacterial Factors

.

Agrobacterium strain:

Strains differ in their virulence and host range (common strains include LBA4404, EHA101, EHA105, GV3101, and AGL1)

.

Plasmid type:

The specific binary vector can influence transformation efficiency

· Bacterial growth phase and density: Log-phase cultures at appropriate densities typically give the best results

.

Induction of virulence genes:

Proper induction of vir genes is essential for efficient T-DNA transfer

Environmental and Cultural Factors

Co-cultivation conditions:

Temperature, duration, light, and medium composition

· Presence of vir-inducing compounds: Phenolic compounds like acetosyringone enhance vir gene expression

Antioxidants:



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Addition of antioxidants can reduce tissue browning and necrosis

Osmotic pressure:

Osmotic stress can sometimes enhance transformation

pH:

Slightly acidic conditions favor vir gene induction

- Plant growth regulators: Affect both transformation susceptibility and regeneration efficiency

Molecular Factors

T-DNA size:

Larger T-DNAs are generally transferred less efficiently

Border sequences:

Integrity of the right and left border sequences is critical.

Molecular Farming

- Production of pharmaceuticals (vaccines, antibodies, therapeutic proteins)
- Production of industrial enzymes and specialty chemicals
- Biofuel production through modified biomass composition

Recent advances in Agrobacterium-mediated transformation include:

Expansion of Host Range

Traditional limitations on transforming certain plant species, particularly monocots

Integration with Genome Editing

The combination of Agrobacterium-mediated transformation with CRISPR/



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Cas9 and other genome editing technologies has revolutionized plant genetic

engineering:

- Delivery of CRISPR components via *Agrobacterium*
- Development of DNA-free genome editing approaches
- Precise targeted modifications rather than random transgene integration
- Multiplex editing for simultaneous modification of multiple genes
- Base editing and prime editing for precise nucleotide changes

Automation and High-Throughput Systems

For large-scale applications, particularly in functional genomics:

- Robotics for automated transformation and screening
- High-throughput vector construction platforms
- Standardized protocols for consistent transformation
- Integration with phenotyping platforms for rapid characterization of transformants

Despite these advances, challenges remain in *Agrobacterium*-mediated transformation, including genotype-dependent transformation efficiencies, difficulties with certain economically important species, transgene silencing and woody plants, have been increasingly overcome through:

- Identification of *Agrobacterium* strains with broader host ranges
- Optimization of co-cultivation conditions for recalcitrant species
- Addition of surfactants, antioxidants, and other compounds to enhance transformation
- Development of tissue-specific protocols for species-dependent optimization



Improved Vector Systems

Modern transformation vectors incorporate numerous refinements:

- Gateway-compatible vectors for rapid cloning
- Multigene transformation vectors for introducing multiple genes simultaneously
- Vectors with site-specific recombination systems for targeted integration
- Vectors with inducible promoters for controlled gene expression
- Vectors with tissue-specific or developmental stage-specific promoters

Marker-Free Systems

Addressing concerns about marker genes (particularly antibiotic resistance genes) in

transgenic plants:

- Co-transformation and segregation approaches to remove marker genes.

Conclusion and Future Perspectives

Plant transformation techniques have evolved significantly since their inception, from rudimentary methods with low efficiencies to sophisticated approaches capable of precise genomic modifications. Each transformation method whether chemical, physical, or biological offers unique advantages and limitations, making them suitable for different applications and plant species. The choice of transformation technique depends on multiple factors including the plant species, the specific research objectives, available resources, and the desired outcome. Agrobacterium-mediated transformation remains the method of choice for many applications due to its relatively high efficiency, tendency to produce single or low-copy transgene insertions, and ability to transfer relatively large DNA segments. However, physical



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methods like particle bombardment continue to be valuable, particularly for species recalcitrant to *Agrobacterium* infection. The future of plant transformation appears promising, with several emerging trends likely to shape developments in the field:

Continued refinement of transformation protocols for recalcitrant species,

particularly economically important crops and orphan crops that have received

less research attention

2. Development of more precise genome modification techniques, moving beyond

random integration to targeted modifications through site-specific recombination and genome editing technologies

3. Increased focus on multigene transformation for engineering complex traits

and metabolic pathways, requiring the transfer and coordinated expression

of multiple genes

4. Advancements in vector design, including tissue-specific, developmentally

regulated, and environmentally responsive promoters for more nuanced control

of transgene expression.

Integration of transformation techniques with high-throughput phenotyping

and multi-omics approaches for more comprehensive evaluation of transformants



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6. Development of transient transformation systems for rapid functional testing

without the need for stable integration and regeneration

7. Exploration of alternative transformation methods, including the use of

engineered nanoparticles as DNA delivery vehicles and exploitation of novel

biological vectors beyond *Agrobacterium*

8. Continued improvements in marker-free transformation systems to address

regulatory concerns and public perceptions regarding transgenic crops

9. Application of machine learning and artificial intelligence to predict transformation success and optimize protocols based on plant genotype and physiological status

10. Greater understanding of the molecular mechanisms underlying successful

transformation, leading to rational design of transformation protocols rather

than empirical optimization.

Summary

Techniques of transformation refer to the various methods used to introduce foreign genetic material into host cells to create genetically modified organisms. These techniques are fundamental in molecular biology, biotechnology, and genetic engineering. Common methods include *Agrobacterium*-mediated transformation, where the natural ability of *Agrobacterium tumefaciens* to transfer DNA into plant cells is exploited; gene gun or biolistic method, which physically delivers DNA-coated particles into target cells; electroporation, where electrical pulses temporarily make cell membranes permeable to DNA; chemical



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methods such as using calcium phosphate or polyethylene glycol to facilitate DNA uptake; and microinjection, which involves directly injecting DNA into the nucleus of a cell. Each method has its advantages and limitations depending on the type of organism, tissue, and desired efficiency. These transformation techniques enable the development of transgenic plants and animals, the production of recombinant proteins, and advancements in research and therapeutic applications.

MCQs: Techniques of Transformation

1. Which of the following is NOT a common method for transforming bacterial cells?

- A) Heat shock
- B) Electroporation
- C) Conjugation
- D) Microinjection

Answer: D) Microinjection

2. Electroporation works by:

- A) Using chemical treatment to increase cell permeability
- B) Applying a high-voltage pulse to create temporary pores in the cell membrane
- C) Introducing DNA through viral infection
- D) Fusing two cells using polyethylene glycol

Answer: B) Applying a high-voltage pulse to create temporary pores in the cell membrane

3. The process of introducing foreign DNA into a plant cell using *Agrobacterium tumefaciens* is called:

- A) Lipofection
- B) *Agrobacterium*-mediated transformation



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- C) Biolistics
- D) Microinjection

Answer: B) Agrobacterium-mediated transformation

4. Which technique involves shooting microscopic particles coated with DNA into plant tissues?

- A) Electroporation
- B) Heat shock
- C) Biolistics (gene gun)
- D) Protoplast fusion

Answer: C) Biolistics (gene gun)

5. Which of the following is true for chemical transformation using calcium chloride?

- A) It requires high-voltage electric pulses
- B) Cells are made competent by chemical treatment to uptake DNA
- C) DNA is directly injected into the nucleus
- D) It works only in plant cells

Answer: B) Cells are made competent by chemical treatment to uptake DNA

6. Which transformation method is commonly used for animal cell lines?

- A) Microinjection
- B) Agrobacterium-mediated transformation
- C) Biolistics
- D) Conjugation

Answer: A) Microinjection

7. Lipofection is a method of transformation that uses:



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- A) Viruses
- B) Lipid-based vesicles to deliver DNA
- C) Electric shock
- D) Heat treatment

Answer: B) Lipid-based vesicles to deliver DNA

8. Protoplast fusion is mainly used for:

- A) Bacterial transformation
- B) Fungal genetic modification
- C) Plant hybridization at the cellular level
- D) Viral gene delivery

Answer: C) Plant hybridization at the cellular level

9. Which of the following factors can increase the efficiency of bacterial transformation?

- A) Using log-phase cells
- B) Increasing DNA concentration
- C) Optimizing heat shock duration
- D) All of the above

Answer: D) All of the above

10. In Agrobacterium-mediated transformation, the foreign gene is delivered into the plant genome through:

- A) T-DNA (Transfer DNA) region of Ti plasmid
- B) Nuclear injection
- C) Viral vector integration
- D) Plasmid replication in the cytoplasm

Answer: A) T-DNA (Transfer DNA) region of Ti plasmid



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short answer type questions on Techniques of Transformation

- Define transformation in the context of molecular biology.
- What is the difference between natural transformation and artificial transformation?
- Name two bacterial species commonly used for transformation experiments.
- What is the role of competent cells in transformation?
- Explain the function of plasmids in bacterial transformation.



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Unit 5.3 : Applications of Plant Tissue Culture

The significant applications of plant tissue culture has significantly altered the dynamics of modern horticulture and agriculture. Plant tissue culture is a powerful biotechnological tool used for the cultivation of plant cells, tissues or organs in artificial nutrient media under sterile conditions, allowing for manipulation and regeneration of plants with desired characteristics. Developed techniques revolutionized conventional plant propagation processes and provided a new approach to crop enhancement, preservation, and production at the market level.

Applications of Plant Tissue Culture in Horticulture and Agriculture

Applications in Horticulture

Micropropagation

The most prominent commercial applications of plant tissue culture are that of micropropagation. One technique that allows elite plant genotypes to be multiplied quickly in vitro to produce genetically identical clones. Micropropagation in the course of several days to a few months can generate thousands or even millions of plants from a single explant, a significant advancement compared to traditional propagation methods. The process systematically includes several processes including aseptic cultures establishment, propagules propagation, root induction, and acclimatization under ex vitro conditions. For ornamental plants that include orchids, anthurium and gerbera, the industry standard has developed micropropagation, which allows for production year-round, breaking the shackles of seasonal dependence when the planting with seeds is concerned. This method is especially useful for species that are hard to grow via the traditional means, species that have long infantile periods or a low seed germination. Micropropagation revolutionized the commercial production of many ornamental species, including the genera roses, chrysanthemums, carnations, and ferns. The technique guarantees



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consistency, minimizes the production cycle, and most importantly, promotes the fast deployment of new cultivars in the marketplace. For example, one orchid protocorm can yield thousands of plants in a matter of months while traditional propagation can take years. As the in vitro cultures are free of pathogen, this technique also allows exchanging germplasm in international conditions while avoiding the quarantine. This has further accelerated the global dissemination of elite horticulture varieties, further fuelling industry growth globally.

The Generation of Pathogen-Free Plants

One of the most widely used and valuable types of tissue culture in horticulture is the production of disease-free plants. Numerous vegetatively propagated crops also suffer from systemic pathogen accumulation over time, leading to yield losses and quality decline. Convenient techniques are available in tissue culture in the form of meristem culture and virus indexing protocols. The apical meristem of plant shoots is often virus-free even in infected plants due to limited vascular Tissue culture also offers advantages in the agricultural sector where elite varieties are in high claim as quality planting materials through rapid multiplication. This is of great benefit to: Vegetatively propagated crops: For crops such as cassava, potato, sweet potato and sugarcane, the tissue culture allows to produce a large number of disease-free planting material. For example, the incorporation of micropropagation in the seed production programs in sugarcane has raised the yield record by 15-30% in many countries owing to healthy and vigorous planate. New varieties released: The availability of planting material is often the bottleneck in the adoption when a new improved variety is released. Tissue culture hastens the multiplication process and thus faster dissemination to farmers. In banana breeding programmes, promising hybrids can be multiplied from a handful of plants to thousands in a year, enabling swift field testing and distribution. Perennial crops: For tree crops with extended juvenile



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periods, tissue culture greatly shortens propagation time. An example of this is date palm micropropagation, where 3-5 years pass from planting offshoots till regular harvesting of date fruits, while tissue culture has been demonstrated to allow for the production of thousands of plants from a single explant, within 18 – 24 months. The use of tissue culture-derived planting material leads to yield increase, the economic effects of this extend far beyond this. Field research in Kenya, Uganda, and Tanzania have shown that farmers growing tissue cultured banana which generally allows increased productivity, uniform maturity, and better access to markets stood to benefit 50-100 percent more profitable as compared to farmers growing using conventional planting material.

Genetic Transformation

The process of plant genetic transformation adding new genes to crop plants to give them new properties is built on top of the techniques of plant tissue culture. Tissue culture is a vital part of this technology, for the successful genetic modification of plants depends on the regeneration of transformed cells into complete, functional plants. Genetic transformation in agriculture has generated transgenic plants of commercial importance through:

.

Bt crops:

Genetically engineered with proteins from the soil bacterium *Bacillus thuringiensis* to defend against pests like the European corn borer, these Bt crops of cotton, maize, and eggplant have led to some drop in pesticide applications and boosted yields in parts of the globe.

Herbicide tolerance

Crops developed to be resistant to broad-spectrum herbicides have streamlined weed management systems, most notably in soybean, maize, and canola.

Disease resistance:



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Transgenic papaya with resistance to Papaya ringspot virus saved the Hawaiian papaya industry from destruction, illustrating the effectiveness of this strategy for control of devastating crop diseases. Genetically modified crops designed for improved nutritional qualities, such as Golden

Rice which is designed to produce beta carotene. In each crop species and variety it is their tissue culture research that set regeneration protocols of all transformable species or varieties. In the past, some crops were considered recalcitrant and resistant to transformation; but thanks to advances in tissue culture techniques, the gamut of crops that can be transformed and benefit from genetic engineering has also increased.

Somatic Embryogenesis & Artificial Seeds

Somatic embryogenesis (SE) the formation of embryo-like structures from somatic cells provides distinct advantages for agricultural use. In contrast to organogenesis, where shoots need to go through a rooting phase, somatic embryos give rise to both shoot and root meristems, making regeneration easier. This technology opens the possibility of creating “artificial seeds” by encapsulating somatic embryos in protective coats and offering a delivery system similar to that of natural seeds. In case of hybrid crops where seed production is costly or complicated, artificial seeds are an alternative propagation method, including hybrid rigidity and enabling mechanical sowing of hybrid crops in the farms. In forestry and plantation crops, somatic embryogenesis is widely used for large-scale multiplication of superior elite genotypes with desired traits like better growth, improved wood quality or enhanced stress resistance. One technique used to commercialize somatic embryogenesis in conifers is with companies like Weyerhaeuser and ArborGen which produces over millions of high value plantlets each year. Somatic Embryogenesis in Bioreactor Systems Especially Suitable for

Production of Haploids and Double Haploids



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Haploid plants can be produced via anther, pollen, or ovule culture, and chromosomes can be doubled afterwards to generate double haploids (DHs), which can transform breeding programs for many agricultural crops. Now this method can produce entirely homozygous lines in just one generation, significantly decreasing the time for cultivar improvement. Connecting the dots DH technology is now a routine breeding tool in crops such as barley, wheat, maize, rice and rapeseed. The technique comes in handy especially for:

Homozygous parental lines

: Developing and producing hybrid seeds

Mutation Bathing:

Immediate expression of its recessive mutations without the effects of dominance

Transformation supply of isogenic material for obtaining a genetic modification

The financial relevance of DH technology is considerable. For example, in European wheat breeding, triumvirate systems have significantly reduced the time for developing Large Scale Propagation Sildenafil Citrate 100mg Tablets Bioreactor Systems verfolgen, which allow an extreme Verf insertion of plants, and thus especially commercial use through RIPPERSCHACHTUNG. This method has been applied commercially in the field of agriculture as it provides higher multiplication rates and genetic fidelity than conventional methods, especially among coffee, oil palm, and rubber.

Accelerated Screening for Large Populations in Small Space

In crops, in vitro selection has produced useful mutants with enhanced traits such as disease resistance, stress tolerance, and quality attributes. One successful study, for instance, involved the selection of salt-tolerant variants of rice and wheat from cell cultures that had been exposed to high sodium concentrations, which performing well in



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saline field conditions. Analogously, disease-resistant mutants have been selected by adding pathogen toxins (or filtrates) to culture media, in which only resistant cells can survive and regenerate. So-called transgenic techniques have resulted in varieties of many crops, including banana, sugarcane, and potato, that are more resistant to certain fungal diseases. Combining in vitro mutagenesis with modern genomics approaches streamlines identification and characterization of beneficial mutations, and continues to be an area of agricultural innovation.

Production of Secondary Metabolites

Culturing plant tissue has many advantages: it allows the production of high value secondary metabolites in a controlled environment, irrespective of soil types and climate zone. This method has augmented significance for the manufacture of agriculturally-relevant compounds including those employed in pharmaceutical, flavors, fragrances, and biopesticides. Metabolite production has been enhanced by developing cell suspension cultures, hairy root cultures and elicitation strategies. Commercial successes include:

- Production of Shikonin from *Lithospermum erythrorhizon* Cell Cultures by Mitsui

Petrochemical Industries

- Taxol (paclitaxel) production from *Taxus* cell cultures
- Ginsenosides production from *Panax ginseng* cultures

Flavours and perfumes at large from plants used in food industry

In vitro production has some benefits for agriculture applications such as uniform quality, absence of environmental contaminants and reduced pressure on wild plant populations. The technology is also useful for producing compounds that are difficult to chemically synthesize or extract from whole plants efficiently. Ongoing advances in metabolic engineering and bioreactor design promise to further



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increase the economic viability of this approach for a broader array of compounds.

Exchange and Quarantine of Germplasm

For this reason, plant tissue culture enables international transfer of plant genetic resources with low phytosanitary risk. As in vitro cultures are free of most pathogens and pests most quarantine steps are eliminated and most the risk of introducing exotic diseases is reduced.

This has important implications for agricultural crops:

- The exotic germplasm is more readily available to breeding programs, thus broadening the region of the genetic base for crop improvement.
- New varieties can be globally dispersed in clean form, accelerating international.

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gene banks back into their native countries.

Genetic variation in rare landraces and primitive cultivars

Apart from slow growth storage and cryopreservation, already mentioned before, tissue culture makes the rescue of endangered crop relatives possible via embryo culture and in vitro propagation. For instance, some wild coffee species at risk of extinction have been saved by tissue culture, keeping genetic resources that might have useful traits for disease resistance or climate adaptation. Significant international efforts, such as the Svalbard Global Seed Vault, are now supplemented by in vitro collections for crops that cannot be preserved successfully as seeds. Various tissue culture-based conservation efforts are also supported by the Global Crop Diversity

Trust, which notes that they play a vital part in ensuring that agricultural diversity is preserved for future generations.

Embryo Rescue and Broad Hybridization

The embryo rescue technique allows the recovery of interspecific and intergeneric hybrids that typically abort due to endosperm failure or other post-zygotic barriers. But by culturing immature embryos on artificial media, breeders can access genetic diversity from wild



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relatives and related species that would otherwise be unavailable via conventional crosses.

This strategy has broadened the gene pool available for crop improvement in many

species:

- In wheat, embryo rescue enabled interspecific transfers of disease resistance

genes from wild Triticum species

- In rice, traits such as bacterial blight resistance and tolerance of sub-optimal

soil conditions have been introduced by crosses with wild relatives.

Crop Improvements Via Double Haploids

Apart from speeding up breeding cycles, double haploid (DH) technology has a number

of particular benefits for crop improvement:

- This is especially important for hybrid breeding, where DH lines provide perfectly homozygous parents to maximize heterosis in the resulting F1 hybrids. This has worked especially well in maize, for which the commercial hybrid seed industry puts greater demands on lines of doubled haploids for parental lines than for any other crop.

- DH lines are frequently used to map complex traits in population genetics studies, and they facilitate genotype-phenotype associations by removing the genetic segregation of the founding parents in true-breeding individuals. This also has hastened the detection and characterization of quantitative trait loci across several crops.

- Mutation detection in DH lines occurs much more rapidly in mutation breeding as recessive mutations do not need to be selfed through many generations. And when combined with in vitro mutagenesis, this



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approach has produced useful variants in crops such as barley and rapeseed.

- Rapid generation of homozygous transgenic lines by successively doubling the

chromosomes of the transgenic haploid cells (microspores or microspore derived cells) bypassing the need of backcrossing/selfing the introduced gene for stabilization, makes the transgenic crop development easy but bumpy due to many factors associated with the haploid cells which are reported to affect the transformation efficiency.

- This trend towards the widespread adoption of DH technology does not only apply to the major cereal crops but also horticultural species such as pepper, eggplant and melon have benefited from improved protocols and this approach is now more accessible to breeders.

Somatic Hybridization and Its Application in Agriculture

Genetic recombination can occur by somatic hybridization via the fusion of protoplasts of sexually incompatible species to produce new combinations of genomes for crop improvement. This novel approach can merge cytoplasmic and nuclear genomes in many different ways, including:

- Symmetric fusion, in which the full genomes of both parents are joined

- Asymmetric fusion, which occurs when only part of one of the parents is

transferred.

- Cybridization, focused on organellar genome transfer without nuclear integration

Examples of commercially valuable results of somatic hybridization in agriculture include:



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- In potato, somatic hybrids between cultivated *Solanum tuberosum* and wild

species have provided late blight resistance and other desirable traits

- Intergeneric somatic hybrids in citrus introduced cold hardiness of trifoliolate

orange together with fruit quality contributions from commercial citrus cultivars

(Graham et al. 1994; Zhang et al. 2017).

- For hybrid seed production systems cytoplasmic male sterility has been

transferred by cybridization in brassica crops

- Although protoplast regeneration of fertile plants available for many crop species

is still elusive, the utility of this method is being extended with continued

methodological advancements.

Certified nurseries or regulatory seed producers

It has been successful particularly for vegetatively propagated crops such as potato, strawberry, cassava and other fruit crops. Many seed certification systems globally rely on nuclear stock programmes as the basis of their potato industries, with pathogen free tissue culture laboratories supplying the first inoculation of pathogen-free material into seed multiplication systems.

There are enormous economic virtues as well:

- Virus-indexed seed programs are estimated to contribute \$300 million a year to the U.S. potato industry through increased productivity.
- in Kenya, virus-free sweet-potato planting material has increased yields by



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30% to 50%

- Clean seed programs for cassava in Brazil avoid up to 60% of loss caused by viral diseases in targeted areas. With climate change and increasing global trade spreading plant pathogens, these

programs become ever more important in securing agricultural productivity and food security. Tissue culture makes it possible to rapidly multiply new elite genotypes, developed or identified under breeding programs to supply adequate planting material. This creates a pipeline, taking new varieties from development to adoption by farmers, addressing a key bottleneck in the impact pathway of agricultural research. This approach is especially useful when:

Varieties that have been recently released from breeding programs

- Farmers-Identified Landraces Selected For Wider Distribution
- Genotypes capable of tolerating stress required to adapt to climate
- Ethylene resistant crops developed to increase shelf life

Here are a few success stories showcasing the application of it:

- Tissue culture multiplication of NERICA rice varieties in Uganda allowed quick

distribution of these higher-yielding, stress-tolerant lines to farmers, speeding up

adoption

- Initiatives to combat iron deficiency through biofortification in India were facilitated

by the quick multiplication of iron-rich pearl millet varieties through tissue culture

Sub-Saharan Africa has seen the scale-up of drought-tolerant maize varietal releases through integrated seed systems, using tissue culture for primary seed multiplication. Integrating tissue culture with conventional seed systems creates efficient delivery channels for better



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varieties and is key to making research results work for farmers and consumers.

In Vitro Crop Improvement

In addition to being a supportive technology to standard breeding, tissue culture also directly plays a role in crop improvement through:

- Somaclonal variation, taking advantage of the native genetic changes that occur

naturally in the tissue culture process

- Chemically or physically inducing mutations on cultures of cells or tissue (in vitro

mutagenesis)

- In vitro selection, a method of subjecting cultures to selective agents such as

pathogens, toxins, or stress factors

- Protoplast cell fusion from various genotypes or species.

Transfer of Cytoplasmic Male Sterility through Somatic Hybridization

Cytoplasmic male sterility (CMS) is a maternally inherited trait that suppresses

pollen generation (Chen et al., 2008), which is useful for hybrid seed production

in many crops. One way to accomplish that is through tissue culture, one strategy

is somatic hybridization by protoplast fusion, to transfer CMS between species

or genotypes, thus bypassing sexual compatibility barriers.



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This strategy has helped in establishing hybrid seed production systems in several crops:

- In rice, introgressions of CMS from wild relatives to elite cultivars through

protoplast fusion

- In brassica vegetables, somatic hybridization has been used to introgress

CMS systems

- Cybridization (fusion resulting in cytoplasmic hybrids) in sunflower has produced novel CMS sources.

Molecular Farming and Biopharmaceuticals

Which leads to say that plant tissue culture is, in fact, a production platform for high

value recombinant proteins, ranging from pharmaceuticals to industrial enzymes and

specialty chemicals. Compared to the microbial or mammalian cell culture systems,

plant-based production holds advantages in:

- Safety (lower likelihood of contamination by human pathogens)
- Efficiency for many sources of protein
- Capability for complicated post-translational modifications
- Scalability with existing agricultural structures

Several tissue culture systems are used for this goal:

- Heterologous production of biopharmaceuticals in bioreactors cell suspension

cultures, especially for secreted proteins



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- Agrobacterium rhizogenes, Hairy root cultures combining high growth rates with genetic stability
- Virus vector-based temporary expression systems in tissue culture plants
- Genetically modified cell lines for plant production of specialized proteins.

Technology transfer and entrepreneurship

In many regions, plant tissue culture catalyzed entrepreneurship and technology transfer resulting in opportunities, particularly for small and medium enterprises. Some of the key drivers sustaining this trend are:

- Compared to other biotechnologies, relatively low initial investment requirement
- Scalability means being able to start small and grow slowly
- Serving local markets with adapted varieties
- One option is to pursue a technical training program through research institutions and development agencies

Examples of successful business models:

- Small-scale tissue culture laboratories for local crops by women's cooperatives in India
- Farmer owned businesses in Kenya that grow banana plants to meet community demand
- University spin-out companies commercialising research innovations.

Summary

Plant tissue culture is a versatile technique in modern agriculture, horticulture, and biotechnology with wide-ranging applications. It allows for the rapid clonal propagation of plants, ensuring uniformity and disease-free stock, which is particularly valuable for rare,



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endangered, or commercially important species. Tissue culture is instrumental in producing genetically modified plants, facilitating the introduction of desirable traits such as pest resistance, improved yield, or stress tolerance. It also enables the conservation of germplasm through in vitro storage and cryopreservation, safeguarding plant biodiversity. Additionally, plant tissue culture supports secondary metabolite production for pharmaceuticals, enhances hybrid development through embryo rescue, and accelerates breeding programs. Its precision, efficiency, and scalability make it a cornerstone technology for sustainable agriculture, crop improvement, and biotechnological research.

Which of the following is a primary application of plant tissue culture?

- A. Mass propagation of plants
- B. Increasing soil fertility
- C. Reducing rainfall dependency
- D. Enhancing photosynthesis

Answer: A. Mass propagation of plants

2. The production of disease-free plants is possible through:

- A. Callus culture
- B. Micropropagation
- C. Somatic embryogenesis
- D. Protoplast fusion

Answer: B. Micropropagation



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3. Which tissue culture technique is used for producing genetically uniform plants?

- A. Somatic hybridization
- B. Organogenesis
- C. Micropropagation
- D. Suspension culture

Answer: C. Micropropagation

4. Somatic hybridization in plant tissue culture is primarily used for:

- A. Producing disease-free plants
- B. Combining traits from two different species
- C. Increasing seed production
- D. Rooting of cuttings

Answer: B. Combining traits from two different species

5. Secondary metabolite production from plant cells in vitro is achieved using:

- A. Callus culture
- B. Suspension culture
- C. Organ culture
- D. Both A and B

Answer: D. Both A and B



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6. Which application of plant tissue culture helps in conservation of endangered plant species?

- A. Synthetic seed production
- B. Micropropagation
- C. Somatic embryogenesis
- D. All of the above

Answer: D. All of the above

7. Synthetic seeds are produced from:

- A. Zygotic embryos
- B. Somatic embryos
- C. Leaf explants
- D. Root tips

Answer: B. Somatic embryos

8. Protoplast culture is important in:

- A. Somatic hybridization
- B. Callus induction
- C. Micropropagation of orchids
- D. Shoot tip culture

Answer: A. Somatic hybridization



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9. Which of the following is NOT a common application of plant tissue culture?

- A. Mass propagation
- B. Production of secondary metabolites
- C. Soil enrichment
- D. Genetic engineering

Answer: C. Soil enrichment

10. Hairy root culture is mainly used for:

- A. Rapid plant propagation
- B. Production of secondary metabolites
- C. Root regeneration
- D. Disease elimination

Answer: B. Production of secondary metabolites

Short-answer type questions

- What is micropropagation, and how is it used in agriculture?
- How does plant tissue culture help in producing disease-free plants?
- Explain somatic hybridization and its significance in crop improvement.
- What is the role of tissue culture in producing transgenic plants?
- How is plant tissue culture used for the conservation of endangered plant species



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Unit 5.4: Edible Vaccines

Introduction to Edible Vaccines

One such exciting new development in the area of immunization is the idea of edible vaccines, which utilize plants and other edible sources as a means to produce and deliver vaccines in one, revolutionary step. This technology was developed in the early 1990s (15) when researchers first showed that it was possible to genetically introduce plants to produce immunogenic proteins. - The idea was born out of the desire to overcome the challenges posed by traditional vaccines that entail cold chain storage and transportation, needle delivery, and expensive manufacturing processes that often preclude the availability of vaccines in poor areas. The edible vaccine saga started with Charles Arntzen and his team at Texas A&M University who were able to express hepatitis B surface antigen in tobacco. This pioneering work established the design principles for the subsequent investigation of programming plants to become biofactories for the manufacture of vaccines. Since then, various plant species have been investigated for use as hosts for vaccine production, including banana, potato, tomato, rice, lettuce, and corn. That concept has grown to also include edible organisms such as algae and yeast, creating additional platforms for vaccine development. Edible vaccines essentially operate at a cross-roads between medical immunology and plant biotechnology, harnessing the ability of plants to express foreign genes and synthesize complex proteins while retaining their immunogenetic integrity. This approach is based on the notion that mucosal immunization with antigens can invoke the mucosal immune system, which is predominantly located in the gut-associated lymphoid tissue (GALT) representing almost half of the immune system in the human body. This mucosal immunity is particularly important for pathogens that enter the body via mucosal surfaces. vaccines against hepatitis B, cholera, rotavirus, and norovirus. Although the rapid evolution of technologies has facilitated the discovery, characterization, and production of a wide variety of edibles, most of them are still largely at the experimental stage with many of



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them facing various regulatory, standardization and perception challenges restricting their pipeline to widespread applications in clinics. If effective, an edible vaccine would help change the current paradigms of vaccine administration and delivery to overcome global health barriers, serving as an inexpensive, heat-stable and needle free alternative that can potentially aid in immunizing people across developing countries where cold chain development is difficult and healthcare access available is limited. Their development epitomises a convergence of agricultural biotechnology and medical science, illustrating the need for interdisciplinary approaches to overcome complex global health challenges.

Mechanism of Edible Vaccines

Edible vaccines are a remarkable example of harnessing genetic modification to achieve an enticing solution to health issues. However, the process is based on the fact that plant cells can be genetically modified to express specific pathogenic antigenic proteins. Most genetic engineering is accomplished using one of several approaches: *Agrobacterium*-mediated transformation, which relies on the natural gene transfer capabilities of this soil bacterium; particle bombardment (biolistics), a process in which gold or tungsten-coated DNA is physically shot into plant cells; or viral vector systems involving the application of modified plant viruses to transfer transgenes. In both cases, foreign DNA encoding vaccine antigens gets integrated into the plant genome. After being successfully transformed, these plant cells are regenerated into whole plants using tissue culture techniques. By using a different promoter in the genetic construct, the transgenic plants can either express the antigenic proteins throughout all of their tissues constitutively or, in specific organs. It also allows targeting expression to edible parts of the plant (e.g. fruits, leaves, seeds and tubers) to facilitate oral consumption. Its post-translational modification machinery aids proper folding and glycosylation of proteins produced within the plant cells, which is crucial for them to retain immunogenicity. After eating, the plant material with the antigenic



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proteins is digested in the gastrointestinal tract. Vaccine antigens are designed to resist the complete.

Advantages of Edible Vaccines

Edible Vaccines are a tremendous step forward in the world of Vaccines, with significant benefits over conventional vaccination initiatives. These strengths cover technical, logistical, economic and social aspects, in a way that directly addresses many of the constraints that affect conventional vaccine systems. From a technical standpoint, perhaps the most exciting benefit is that it eliminates the need for cold chain. Conventional vaccines almost universally require uninterrupted refrigeration from the moment they are made to the moment they are given, a Herculean task in places with erratic electricity or little refrigeration infrastructure. Edible vaccines, especially as expressed in shelf-stable plant parts (such as seeds or dried fruits), can last at ambient temperatures for long periods of time. This along with thermal stability greatly diminishes any risk of vaccine spoiling and maintains potency without the need for sophisticated storage facilities, making them especially useful in remote or resource limited settings. Another benefit of oral vaccination is the ability to deliver edible vaccines without the need for needles. Do away with injection associated risks like a needle-stick injury, transmission of blood-borne pathogens due to needle reuse, improper sharps disposal, edible vaccines are safer for healthcare workers to administer and recipients to receive. This oral delivery method is great for areas with poor healthcare access and a lack of medical waste management systems. Furthermore, the use of edible vaccines, because of their innocuous and non-invasive delivery mechanism, could also be used to increase compliance rates of vaccination, especially in children and people suffering from needle phobia, which may boost immunization coverage in resistant populations. From a production perspective, edible vaccines capitalize on the natural scalability of agricultural systems. Plants do this very well, acting as bioreactors that harness solar energy, atmospheric carbon dioxide and simple nutrients to generate complex biological



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molecules while also circumventing the high-level fermentation equipment and sterile factories that need to be used in the production of traditional vaccines. Moreover, this scale-up is entirely agnostic to the photoautotrophic host, providing near boundless scalability via conventional agricultural methods and therefore making possible a faster response to a newly emerging infectious disease through the large scale cultivation of vaccine-expressing autotrophic hosts. Edible vaccines can be The relatively cheap production using existing agricultural infrastructure can significantly lower manufacturing costs, compared to traditional vaccines grown in bioreactors or cell culture systems. Moreover, purification requirements, cold chain infrastructure, and dedicated administration equipment are removed from the cost of IM programs. Such major economic advantages could significantly enhance vaccine availability in

lower resource settings where price often is a crippling barrier to full immunization coverage. The elicitation of mucosal immunity represents a unique immunological benefit of edible vaccines. These eliciting mucosal and systemic immune responses through targeting the gut-associated lymphoid tissue. This combined immunity is especially important against pathogens that primarily enter through mucosal surfaces, like respiratory and enteric pathogens. Secretory IgA antibodies can prevent the spread of pathogens at mucosal interfaces, which can give superior protection compared to injectable vaccines that show mainly systemic immunity. From the standpoint of practical implementation, edible vaccines make the logistics of mass immunization campaigns easier. Removing the need for specialized and trained medical personnel to administer these treatments means they can be distributed more widely in non-medical settings, like schools or community centres or even going through food distribution networks. Such administrators could greatly expand the potential reach of vaccination programs to underserved populations with little access to healthcare facilities. Another technical distinction is the potential for multivalent vaccine development. Consider that plants can also be



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engineered to express multiple antigens from different pathogens at once, creating polyvalent edible vaccines capable of conferring protection against multiple diseases in a single dose. This strategy might decrease the number of immunization visits needed and streamline vaccination schedules, thereby enhancing compliance and coverage rates. One of the major ecological benefits of edible vaccines is environmental sustainability. Compared to traditional vaccine manufacturing facilities that generally use more energy and produce more waste, plant-based production systems have significantly lower environmental impacts. Moreover, edible vaccines generate limited medical waste, reducing the environmental impact caused by the construction of non-degradable syringes, needles and packaging materials compared to conventional vaccines. In addition, edible vaccines are safer than conventional vaccines because they avoid the risks of attenuated or inactive pathogens in the traditional vaccines. Only antigenic proteins of interest are expressed in plants so there is no risk of reversion to virulence, which sometimes happen with live-attenuated vaccines. Because of this built-in safety feature, there might be a lower rate of adverse reactions to vaccines and higher public confidence in vaccination programs. Edible vaccines have specific accessibility advantages, especially in developing countries for certain demographics. Importantly, they can be integrated into those indigenous crops that already belong to the local diet, thus promoting cultural acceptability and integration in the current food consumption system. This is culturally compatible, which may lead to higher adoption rates than for less familiar medical procedures. Decentralized-producing capability is a strategic asset for global health security. Developing countries might grow their own vaccine-producing plants, which could lessen their reliance on vaccine imports and bolster the sustainability of their national immunization programs. By ensuring local production capacity, it can boost epidemic preparedness and response capability especially useful during a global health emergency where vaccine nationalism often limits supply to less wealthy countries. Last but not least, edible vaccines allow passive immunization of



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nursing infants. Some of the antigenic proteins or globally produced antibodies can be sucked through the

breast milk when lactating mothers consume those proteins, to develop protective immunity among infants who are otherwise too young to be vaccinated. This distinctive benefit meets a major window of vulnerability in early infancy when traditional maternal vaccination strategies may not be optimal or contraindicated. In summary, edible vaccines portray a multi-dimensional spectrum of benefits which together contribute to overcoming most of the drawbacks of traditional vaccination example. Although challenges associated with standardization, dosage control, and regulatory ranging need greater resolution, the prospective benefits of this novel technology necessitates further exploration and development efforts especially for filling immunization gaps in resource-limited settings that have significant implementation challenges for current vaccination programs.

Summary

The production of transgenic plants involves the introduction of foreign genes into a plant's genome to develop new traits such as pest resistance, herbicide tolerance, improved nutritional content, or enhanced stress tolerance. This process begins with the identification and isolation of the desired gene, followed by its insertion into the plant using techniques like *Agrobacterium*-mediated transformation or gene gun methods. Once introduced, the gene integrates into the plant's DNA, and the plant is regenerated through tissue culture. Transgenic plants undergo testing and selection to ensure successful gene expression and desired trait development. This biotechnological advancement has revolutionized agriculture by improving crop yield, reducing chemical use, and contributing to food security.



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MCQs:

1. What is the process of creating transgenic plants?

- a) Genetic modification through traditional breeding
- b) Introduction of foreign genes into plant cells
- c) Hybridization of plants from different species
- d) Both b and c

2. Which technique is commonly used to transfer foreign genes into plants?

- a) Electroporation
- b) Agrobacterium-mediated transformation
- c) Virus-mediated transformation
- d) Both b and c

3. What is the role of the Ti plasmid in Agrobacterium-mediated transformation?

- a) It carries the foreign genes into the plant cell
- b) It induces disease in the plant
- c) It controls plant growth
- d) It helps in regenerating tissues

4. What is a common method for selecting transgenic plants?

- a) Genetic fingerprinting
- b) Antibiotic selection
- c) Random mutation
- d) Both b and c

5. What is the purpose of edible vaccines in plants?



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- a) To produce antibodies
- b) To protect plants from pests
- c) To prevent diseases in humans
- d) To improve plant growth

6. Which transformation method uses a gene gun?

- a) Electroporation
- b) Agrobacterium-mediated transformation
- c) Particle bombardment
- d) Chemical transformation

7. What is the advantage of transgenic plants over traditional plant breeding methods?

- a) They have better disease resistance
- b) They grow faster
- c) They are more genetically diverse
- d) All of the above

8. Which of the following is an application of transgenic plants in agriculture?

- a) Pest resistance
- b) Drought resistance
- c) Nutritional enhancement
- d) All of the above

9. What is the main advantage of edible vaccines over traditional vaccines?

- a) They are more effective



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- b) They are easier to produce and distribute
- c) They require fewer resources
- d) All of the above

10. What is a major challenge in producing transgenic plants?

- a) Genetic instability
- b) High production cost
- c) Public acceptance
- d) All of the above

Short Questions:

1. Define transgenic plants.
2. List the methods of producing transgenic plants.
3. What are the steps involved in Agrobacterium-mediated transformation?
4. Explain the concept of edible vaccines.
5. How is selection of transgenic plants achieved?
6. Describe the role of the Ti plasmid in genetic transformation.
7. What is the importance of transgenic plants in agricultural research?
8. How does chemical transformation work in plant genetic modification?
9. What are the advantages of using edible vaccines for disease prevention?
10. Discuss the applications of plant tissue culture in genetic engineering.



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