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Plant Tissue Culture

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

SELF LEARNING MATERIAL

DSCC407

PLANT TISSUE CULTURE

MATS University

PLANT TISSUE CULTURE

CODE: OLD/MSS/BSCB/407

Reference

COURSE DEVELOPMENT EXPERT COMMITTEE

1. Prof. (Dr.) Vishwaprakash Roy, School of Sciences, MATS University, Raipur, Chhattisgarh
2. Dr. Prashant Mundeja, Professor, School of Sciences, MATS University, Raipur, Chhattisgarh
3. Dr. Sandhyarani Panda, Professor, School of Sciences, MATS University, Raipur, Chhattisgarh
4. Mr. Y. C. Rao, Company Secretary, Godavari Group, Raipur, Chhattisgarh

COURSE COORDINATOR

Dr.Prashant Mundeja, Professor, School of Sciences, MATS University, Raipur,Chhattisgarh

COURSE /BLOCK PREPARATION

Dr.Meghana Shrivastava, Associate Professor,School of Sciences, MATS University, Raipur, Chhattisgarh

March, 2025

FIRST EDITION: 2025
ISBN: 978-93-49916-64-7

Printed & Published on behalf of MATS University, Village-Gullu, Aarang, Raipur by Mr. Meghanadhudu Katabathuni, Facilities & Operations, MATS University, Raipur (C.G.)

Printed at: The Digital Press, Krishna Complex, Raipur-492001(Chhattisgarh)

Acknowledgements:

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MODULE INTRODUCTION

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

Contents

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 01: Plant Tissue Culture

Introduction, Terms, and Definitions

Plant tissue culture is the most important advancement in modern plant science, paving the way for plant propagation, conservation, as well as improvement. Basically plant tissue culture is an ex vitro growing of plant cells, tissues or organs in an artificial nutrient medium under aseptic conditions. Plant tissue culture (also known as micropropagation) emerged in the early 20th century, and Austrian botanist Gottlieb Haberlandt is commonly regarded as its founder, due to groundbreaking experiments in 1902. Haberlandt's initial attempts to culture isolated plant cells had proved unsuccessful, but his vision set the stage for later advances in this area. At the core of plant tissue culture lies the concept of totipotency, the extraordinary potential of plant cells to develop into full-fledged plants. However, unlike animal cells which lose this potential often in their life cycle, the vast majority of plant cells maintain the genetic potential to differentiate into any type of cell—which means they can act as stem cells. This plasticity allows researchers to produce entire plants from single cells, small tissue fragments, and specialized organs. The last permissible developmental state of a cell is called totipotency, so when we refer to the totipotent states of embryonic (pluripotent) stem cells, we are following the pioneering work of F.C. Steward in the 1950s in his famous carrot experiments, in which Steward was able to regenerate whole carrot plants from single phloem cells. To have better understanding of discipline of plant tissue culture, we must know few terminologies. In tissue culturing, the "explant" is the original plant tissue removed from the parent plant. This could be a shoot tip, leaf segment, root section, or even a few individual cells. The nutritive solution refers to the specific medium that provides the macro and micronutrients, vitamins, plant growth regulators, and sources of carbon (usually in the form of sucrose) necessary for embedded plant tissue growth along with undefined products like coconut milk and yeast extract. This medium supplies everything that a plant needs, even in the absence of soil. Callus means an unorganized

proliferation of cells formed after plant tissues are cultured on the appropriate media and is generally called an intermediate stage of regeneration plan. The term "organogenesis" refers to the development of shoot, root and leaf organs from cultured tissues, while "somatic embryogenesis" refers to the formation of developing embryo-like structures from somatic (i.e., non-reproductive) cells. "Micropropagation" refers to the fast vegetative replication of plants-a tissue culture technique. Plant growth regulators (PGRs) are an important input in tissue culture, directing cellular differentiation and development. These PGRs include auxins (e.g., 2, 4-D, NAA, IAA), which promote root formation and cell elongation; cytokinins (e.g., kinetin, BAP, zeatin), which promote cell division and shoot formation; and gibberellins, which promote stem elongation and breaking dormancy. These auxins and cytokinins not only play important roles in organogenesis, but the balance and ratio of them also play a critical role in the development trajectory of the cultured tissues. You have to have a tightly controlled environment for the culture. Culture conditions (for example, temperature [usually 22-28°C], light levels and period [often 16 h light/8 h dark cycle], humidity, and air composition) can have profound effects on cultures success. Cultures are usually kept in growth chambers or some kind of rooms where those environmental factors can be controlled tightly. One of the most important features of plant tissue culture is maintaining an aseptic(sterile) environment which is applied during the whole process. The medium used for growing plant cultures is nutrient-rich, which promotes microbial growth, leading to microbial contamination of the plants. These aseptic techniques encompass the sterilization of equipment, media, and plant material as well as performing work in laminar flow cabinets that deliver filtered, particle-free air. Plant tissue culture is used in many aspects of plant science and agriculture. In plant breeding, it enables approaches such as embryo rescue to bridge hybridization barriers between species. In conservation geneticists aid the preservation of endangered species through germplasm storage. In commercial horticulture, it enables the rapid, inidivids progression of the elite, disease free plantlets. Biotechnology has put forth a lot of new applications either in genetic transformation, secondary metabolites production, and virus free plants development via meristem culture.

Types of Culture

There are many types of culture involved in plant tissue culture, and each one has a different process and purpose. Recognizing these diversified culture systems is key to successfully employing the plant tissue culture methods in basic research and commercial applications.

Callus Cultur: Classical Plant Tissue Culture Types In this method, high auxin concentrations induce callus (undifferentiated cell masses) from explants on solid media containing appropriate plant growth regulators. The callus obtained is an unorganized mass of parenchymatous cells in the form of friable amorphous body. Callus cultures are used for several purposes, both as a source of material for cell suspension cultures and an intermediate plant regeneration system, as well as for the target of genetic transformation. The cellular composition of callus is highly dependent on explant source, culture conditions, and plant species. Some callus cultures are genetically stable, while others may show somaclonal variation, which are genetic or epigenetic changes that emerge during the culture process. Although this variability can present issues for clonal propagation, it can also result in the emergence of novel traits of agricultural or horticultural significance.

Cell Suspension Culture: plant cells are cultured as an agitated suspension in liquid medium, enabling the cells to proliferate. Such cultures are generally established by transferring friable callus into liquid medium and then kept on orbital shakers for aeration and cell aggregation prevention. The use of cell suspension cultures has advantages such as rapid growth rates, homogeneity within each culture, and ease of scaling. They have proved especially useful for biochemical and physiological studies, generation of secondary metabolites and for starting material for protoplast isolation. Commercial production of high-value compounds such as shikonin (derived from Lithospermum erythrorhizon), ginsenosides (from Panax ginseng), and paclitaxel (from Taxus species) requires specialized systems for the large-scale cultivation of plant cell suspensions. Suspension cultures grow according to a sigmoidal curve, including lag, exponential and stationary phases, which requires frequent subculturing to keep them alive.

Protoplast Culture: Split cells are cultured; the cell walls are removed from cells, usually by enzymatic digestion with cellulases and pectinases. These naked cells, sans

their plasma membranes, can be extracted from multiple tissues or from cell suspension cultures. Protoplast culture requires stringent conditions, such as osmotic pressure on the medium that must be strictly controlled to avoid cell lysis. Autotrophic conditions for protoplast culture are relatively important for somatic hybridization (recombination of protoplasts of various species) and genetic transformation. If the protoplasts are successfully cultured, they can regenerate their cell walls, divide, and ultimately regenerate into whole plants. Nevertheless, protoplast culture is technically demanding, with regeneration ability differing markedly among species, relatively simple in some Solanaceous plants, such as tobacco, but exceedingly difficult in cereals and many woody species.

Anther and Microspore Culture: refers to the specific type of culture used for the generation of haploid plants. The other approach is to culture intact or isolated microspores (immature pollen grains) in the anthers, causing them to undergo embryogenesis without going through the normal gametophytic development. Since haploids can be recovered from these matured haploid embryos, it is possible to regenerate haploid plants which can be subsequently subjected to either spontaneous chromosome duplication or treated with colchicine, leading to the recovery of doubled haploids (completely homozygous).. This method enables plant breeders to achieve in a single generation what other approaches would take several generations of inbreeding to accomplish, thus dramatically speeding up breeding efforts. Success is influenced by the developmental stage of the microspores (late uninucleate to early binucleate stage generally optimal), genotype, pretreatment conditions (cold or heat shock often promoting androgenesis), and culture medium composition. The anther culture is effective in crops like rice, wheat, barley, and a few Brassica species.

Embryo Culture: is the in vitro cultivation of excised embryos, from zygotic embryos up to mature embryos. This technique plays multiple important roles; it can save from abortion embryos of broad crosses that would abort due to endosperm failure (embryo rescue), break dormancy of seeds, shorten breeding cycles by germinating immature embryos, and provide a system for studying embryonic development. The culture requirements differ dramatically based on the embryonic developmental stage (the younger the embryo, the larger the media complexity, higher sucrose concentrations, potential growth regulators, etc.). In crops such as wheat, barley, cotton, and a wide

range of fruit species, embryo culture has proved to be a vital tool in broadening the gene pool available to plant breeders through the process of interspecific and intergeneric hybridization.

Meristem Culture: This is a method of culturing apical or axillary meristems (dome-shaped portions of actively dividing cells located at the tips of roots and axillary buds). This is a powerful procedure for generating virus-free plants since meristematic regions are often non-infected, even when plants are systemically infected, due to the lack of developed vascular systems and high metabolic activity. Meristem culture: This commonly involves the excision of small (0.1-0.5 mm) meristematic domes (harvested along with 1–2 leaf primordia) followed by culturing on appropriate media. The combination of meristem culture with thermotherapy (high-tempe-ra-ture treatment of donor plants) has been found to be quite effective tO increase the success of virus elimination. It has been commercialized for the production of virus-free planting material of hundreds of crops, including potato, sweet potato, cassava and numerous fruit and ornamental species.

Organ Culture: is the cultivation of isolated plant organs (roots, shoots, leaves, flowers, or fruits) under in vitro conditions. In contrast to callus or cell cultures, organ cultures retain their organizational integrity and structural relationships. For example, root culture is the growth of isolated roots on media, often supplemented with auxins that allows for studies of root physiology, mycorrhizal associations, and root-specific metabolite production. Some of the most common in vitro production systems are shoot culture (to induce shoots tips or nodal segments, mainly for micropropagation); Other organ specific cultures include ovary and ovule culture (for embryological studies and embryo rescue) and anther culture (as described before), and even culture of entire flower. All organs need culture environments that mirror their distinctive physiological needs.

Micropropagation is the most applied and widely used type of plant tissue culture performed at a very high rate vegetative propagation of the plants under aseptic conditions. Although a procedure rather than type of culture, micropropagation usually involves four distinct phases: establishment of aseptic cultures (Stage I), propagation of propagules via synergetic axillary branching or adventitious shoot formation (Stage

II), rooting of developed sprouts (Stage III) and acclimatization of plantlets to ex vitro conditions (Stage IV). Micropropagation has many benefits over traditional propagation techniques, such as high multiplication rates, production of disease-free materials, space efficiency, independence from seasons and clonal fidelity. Their commercial applications are numerous, ranging from forestry (elite tree propagation) to horticulture (ornamentals, fruit crops) to agriculture (bananas, sugarcane, potatoes). Somatic Embryogenesis The formation of embryo-like structures from somatic (non-sexual) cells. In contrast to zygotic embryos that are formed following fertilization, somatic embryos originate from vegetative cells that undergo cellular reprogramming to obtain embryogenic competence. Somatic embryogenesis may proceed either directly from explanted tissues or indirectly through an intermediate callus phase. This usually requires some specific hormonal signals, usually high auxin concentrations for induction, followed by a decrease in auxins concentration for embryo maturation. Somatic embryos follow the same developmental stages as zygotic embryos (the globular, heart, torpedo, and cotyledonary stages in dicots), but are devoid of seed coats and endosperm. This technique has many advantages for mass propagation as follows: high multiplication rates, possibility of automation (bioreactor systems), and the ability to produce artificial seeds by encapsulating somatic embryos. Somatic embryogenesis is being commercially applied to the propagation of species such as coffee, oil palm and some conifers. Protoplast Fusion and Somatic Hybridization is another example of an advanced application of protoplast culture. The approach involves the fusion of protoplasts of distinct species or genotype using polyethylene glycol (PEG) treatment or electrical stimulation (electrofusion). These hybrid cells can then regenerate into hybrid plants with genetic material from both parents. Somatic hybridization can overcome sexual incompatibility barriers and thus have the potential to produce new gene combinations that cannot be achieved by conventional breeding. This method has been effective in producing intergeneric hybrids across multiple plant families, especially Solanaceae and Brassicaceae. Another method, asymmetric hybridization (between normal protoplasts and protoplasts with inactivated nuclear genomes (by radiation or chemical treatment)), involves introgression under much more controlled conditions. Somatic hybrids, namely, cybrids (with nucleus of one parent but cytoplasmic organelles of both parents) and a range of symmetric and asymmetric hybrids with agriculture importance are examples of its products. Hairy

Root Culture is a particular culture system developed by the infection of the plant with a soil bacterium, Agrobacterium rhizogenes, which integrates certain genes from its root-inducing (Ri) plasmid into some plant cells. The altered cells form highly branched roots with specific growth features: the rapid growth, genetic and biochemical stability, growth in the absence of hormones, and high production of secondary metabolites. They grow in hormone-free media and can be subcultured indefinitely through root tips. This species have their specific values because of their production of secondary metabolites from the roots like the alkaloids, terpenoids, and phenolic compounds. All the commercial plant tissue culture applications include producing pharmaceutical compounds (scopolamine, ginsenosides, etc.) and different kind of medicinal plant metabolites. Furthermore, hairy root cultures are valuable model systems for investigating root biology, plant-microbe interactions and metabolic engineering. All these types of culture are just specific applications of the basic principles of plant tissue culture and related to specific types of research or commercial application. The choice of the right culture system is influenced by species plant, end product, and available technical skills. The modern plant systems have developed through an increased and better integration of plant tissue culture technologies with state-of-the-art molecular tools, bioreactor technologies, and computational methods, providing broader applications in plant science, agriculture, horticulture, and biotechnology.

Aseptic Techniques

Aseptic techniques are essential for successful plant tissue culture, as cultures can be destroyed quickly by microbial contamination. With the media used in plant tissue culture being highly nutrient-rich and incubation conditions characterized by high humidity and moderate temperatures, both promote high microbial reproduction rates. Therefore, strict adherence to aseptic principles is not only desirable, it is essential during all phases of the tissue culture process. Hence having a proper design and upkeep of the culture survive, is considered the basis of aseptic work. Most tissue culture laboratories consist of separate sections, such as media preparation, sterilization, culture manipulation, and incubation. Having these different functions physically separated helps reduce the risk of contamination. The area of manipulation where cultures are initiated and transferred is the most important area. This is typically

equipped with laminar flow cabinets, which blow HEPA-filtered, particle-free air across the work surface horizontally (horizontal laminar flow, as shown in the figure below) or vertically (vertical laminar flow). These cabinets provide a sterile work environment by establishing a constant flow of filtered air in a unidirectional pattern preventing contaminants from entering the work area. The cabinet work surface should be wiped with 70% ethanol before use, and UV sterilization may be performed between work sessions to further reduce microbial loads. The success of laminar flow systems is dependent on their maintenance, including airflow, and filter replacement and calibration. Another key aspect of aseptic technique is the sterility of culture media. The sterilization of media is usually done by autoclaving steam sterilization under pressure at 121°C and 15 psi pressure for 15-20 min. This method is especially good at eliminating bacteria, fungi, and their spores. Some components of the media may heat-labile, such as certain vitamins, plant growth regulators (especially gibberellins and zeatin), and undefined additives (e.g., fruit juices). These components need to be filter-sterilized in membrane filters (usually with pores of 0.22 ìm size) and added to the autoclaved base media when the medium has cooled down to about 50°C, and the sterilized media are usually dispensed into pre-sterilized culture vessels, either test tubes or jars or special containers under aseptic conditions. Media preparation areas should be kept scrupulously clean and all glassware and utensils thoroughly washed and dried prior to sterilization. Plant material preparation is probably among the most difficult step of maintaining aseptic conditions since plant tissues contain a lot of microorganisms on the surface and may harbor some microorganisms internally. This means that, ideally, the procedures used for surface sterilization must be strong enough to kill any contaminants but not so strong that they damage the plant tissues. The general protocol is to wash the explant material externally in running tap water to remove soil particles and minimize the initial microbial load. Next comes immersion in a disinfectant solution typically sodium hypochlorite (commercial bleach), calcium hypochlorite, mercury chloride, hydrogen peroxide or ethanol. Depending on the type of plant tissue, woody explants generally require more rigorous treatments than herbaceous materials and more sensitive tissues like meristems need gentler methods than robust tissues like dormant buds, determining the concentration, contact time, and the disinfectant to use. A common surface sterilization sequence would involve: (1) washing explants under running tap water for 20-30 min; (2) short 70% ethanol

wash for 30 s – 1 min; (3) treatment with 0.5-2.0% sodium hypochlorite solution (a few drops of a surfactant (e.g., Tween-20) could be added to increase efficiency of contact) for 5-20 min; and (4) rinses three to five times in sterile distilled water to wash off the disinfectant completely. All these steps, starting from the disinfectant treatment, must be carried out in aseptic conditions. Other sterilizing agents include mercuric chloride (0.1-0.2%, very effective but environmentally unsound), hydrogen peroxide (3-12%), silver nitrate (1-2%), and numerous commercial disinfectants. Plant material collected from field conditions is usually contaminated by more loads than the one grown in the greenhouse, and pretreatments of source plants with fungicides or bactericides few days behind explant collection can substantially reduce contamination rates.

Sterilization of instruments is another important aspect of aseptic technique. Instruments to dissect with, such as scalpels, forceps, and scissors, must be sterilized before use and repeatedly during long work sessions. The most common method capacitate is flame sterilization, whereby instruments are immersed in 95% ethanol and passed by a flame until the alcohol is combustion. This process can also be repeated frequently during the culture manipulations (after handling each individual explant preferably). Another sterilization method includes hot bead sterilizers, with a constant temperature of about 250°C and a sterilization time of 15-30 seconds once inserted into the beads. In laboratories that are prohibited from using gas flames due to safety protocols, chemical sterilization via soaking instruments may be used, although these methods are generally less effective than methods that thermally induce sterilization. Infection control measures, one commonly known example being personal aseptic discipline, are often underappreciated when it comes to avoiding contamination. Wear clean laboratory coats and tie back long hair. Regular hand washing with antimicrobial soap beforehand is mandatory, and in some protocols, hand disinfection with 70% ethanol is also recommended. The reason why talking, coughing or sneezing is something you want to avoid when working in laminar flow cabinets, is because these actions can potentially result in contaminants entering the sterile workspace. Quiet and slow movement in and around the cabinet will limit air turbulence that could disrupt the laminar flow pattern. Work should go from sterile materials to less sterile components not the other way around. Especially in facilities that do not utilize proper

air filtration systems, the laboratory space itself can act as a source of contamination. Clean the air conditioning filters and maintain minimum foot traffic in culture rooms. Design of culture vessels and procedures for their handling have considerable effect on contamination rates. Tightly sealed closures cotton plugs, aluminum foil, plastic caps, specialized breathable films should be used on vessels. As such, these closures need to establish a barrier to microbiological ingress whilst permitting adequate gas exchange. For transfer and subculturing procedures, culture vessels should be opened only as necessary and closed soon after those manipulations have been performed. The mouth of the vessel should be flamed briefly after open and before closing whenever possible. When moving cultures from vessel to vessel, instruments should not touch the edges of containers, where contaminants may reside. Sharp attentiveness to these procedural particulars greatly mitigates contamination events. Another important aspect of an effective aseptic system is monitoring and detection of contamination. Obvious contamination, such as in bacterial-fungal infestation, can be suspected on visual inspection of cultures daily, where the media may appear cloudy, colonies evident, or if unusual smells are detected. Some contaminants, however, can remain dormant or otherwise display little visible signs of activity, especially in their earlier stages. Nutrient-rich specialized detection media (peptone or yeast extract) can encourage the rapid growth of latent contamination that may remain suppressed in standard plant culture media. Methods such as PCR-based molecular detection provide highly sensitive alternatives for detection of specific contaminants but are mainly used in research rather than routine culturing operations.

Antibiotics and antimicrobials are sometimes employed as adjunct strategies for contamination control; they are not a substitute for proper aseptic technique. Plant culture media can be supplemented with broad-spectrum antibiotics, which include rifampicin, streptomycin, or gentamicin to combat bacterial contamination or fungicides that comprise nystatin or carbendazim for fungal control. However, such additives might have negative effects on plant growth and development, and their frequent use can lead to the emergence of resistant microorganisms. As a result, antimicrobials should be used sparingly, in the interest of conserving precious germplasm from contaminated cultures, rather than as a matter of course. The design of micropropagation systems is becoming more and more adapted to the aseptic operation

conditions. Automation with little human interaction minimises contamination risks with manual handling. Using temporary immersion systems in which plant material is only intermittently in contact with liquid medium may be a way to attenuate the conducive to microbial proliferation conditions of continuous-liquid culture. Physical separation methods, including membrane rafts that separate plant material from the substrate, pose additional barriers to the transfer of contamination. Positive pressure clean rooms with air locks and (in commercial settings) specialized ventilation systems offer multi-facet environmental control beyond that of laminar flow cabinets. Training and quality control practices are essential components of aseptic processes on an organization level, especially in commercial or high-throughput research establishments. Introductory policy for new staff includes structured training in aseptic techniques, and periodic monitoring of contamination rates can identify those who may require further coaching. Documentation and adherence to standard operating procedures (SOPs) Environmental parameters—such as air quality, surface cleanliness, and water purity—are monitored, generating data that can help aseptic systems improve continuously. Regular microbiological testing of water sources, media samples, and environmental surfaces can also detect sources of contamination before they develop into wider problems. The aseptic technique used in plant tissue culture is a holistic system: it is not a collection of separate actions. It includes everything from lab layout, to instrument upkeep, to preparing materials, to operating procedures, to quality assurance. Although there are some variations between different facilities, the basic principles are the same: decrease the amount of sterile items which are exposed to possible contamination, adequately sterilize culture materials to remove microorganisms, and ensure that aseptic techniques are always carried out correctly throughout all culture tasks. Understanding these principles is a critical, pre-requisite knowledge base for successful plant tissue culture practices spanning research, conservation and commercial applications.

Unit 02: Tissue Culture Media

A crucial element of modern biotechnology is plant tissue culture, which is fundamentally dependent on specialized media containing all the nutrients and growth regulators required for cellular growth and differentiation. This type of media acts as

an artificial environment that simulates natural state while maintaining a high degree of control of these factors: physical, chemical, and nutritional that affect plant development. The constituent, types, and recent developments of tissue culture media are important aspects of research that have impactful ramifications for agriculture, conservation, pharmaceutical production, and fundamental plant science.

Composition of Tissue Culture Media

Different types of mass quantities, macro and microelements, minerals, vitamins, vitamins, amino acids, and carbohydrates play a key role in supporting plant growth and morphogenesis, and these are the different things that are formulated in the form of tissue culture media. Together, these components function synergistically to establish an ideal milieu for cellular division, differentiation, and regeneration.

Macronutrients

Macronutrients, which are required in relatively large quantities, form the backbone of tissue culture media, and they include the essential elements nitrogen, phosphorus, potassium, calcium, magnesium, and sulfur. Nitrogen, usually provided as nitrate (NO*f* {) and ammonium (NH,, z) in solution, is a constituent of proteins, nucleic acids, and chlorophyll. Nitrogen form ratios can greatly influence culture performance, where high concentration of ammonium can be limiting a growth in some species. Phosphorus in the form of phosphates is critical for energy transfer in ATP and is found in nucleic acids and phospholipids. Potassium acts as an enzyme activator, and osmotic regulator, Calcium strengthens cell walls, regulates membrane permeability and acts as a secondary messenger in many physiological functions. Magnesium is a vital atom in chlorophyll molecules, as well as activating many enzymes for respiration and photosynthesis. Its incorporation as sulfates forms amino acids such as cysteine and methionine, factors for the structure and function of proteins. The right quantities of these macronutrients are crucial, as improper ratios can cause nutritional inadequate nutria disorders, abnormal growth, or culture failure. Media formulations typically vary not only with the demands of the particular plant species but also with the ontogenetic stage under investigation. Higher nitrogen concentrations, for example,

are beneficial for shoot proliferation, whereas lower nitrogen levels may be optimal for root induction.

Micronutrients

Micronutrients are only needed in minute quantities, but have a great effect on plant metabolism and growth. These are iron, manganese, zinc, boron, copper, molybdenum, cobalt, iodine and sometimes nickel. Iron, typically supplied as one of a variety of chelated forms, such as Fe-EDTA to improve overall availability, is involved in electron transport chains and is a cofactor for many enzymes. Manganese functions as an activator of various enzymes involved in photosynthesis, respiration, and nitrogen metabolism. Zinc is an enzyme activator and involved in auxin synthesis, and boron is involved in cell wall formation and membrane integrity. Copper plays a component of redox enzymes and regulates lignification. D. Nitrogen: Molybdenum serves as a cofactor for nitrate reductase, an enzyme involved in nitrogen metabolism. While needed in minute amounts, deficiencies in these micronutrients can drastically inhibit growth, causing specific symptoms that impact culture success. Micronutrients should also be carefully delivered as they may precipitate or complex, reducing bioavailability. Functional chelating agents, such as EDTA, are used in many contemporary media to keep micronutrients in solution for the culture duration, allowing a constant supply to developing tissues.

Vitamins

Vitamins are cofactors in reactions responsible for metabolic pathways required for plant growth. Mature plants synthesize the vitamins they require, but the biosynthetic capacity of cultured tissues is often limited, and supplementation with exogenous vitamins is required. Among the B vitamins, thiamine, also known as B , is the most important for tissue cultures as it is involved in carbohydrate metabolism and functions as a cofactor for multiple decarboxylase enzymes. Nicotinic acid (niacin) & pyridoxine (B†) – involved in nitrogen metabolism & protein synthesis. Myo-inositol, which is not a vitamin in the strictest sense but is often included in this grouping because of its function in a variety of cell signaling pathways and membrane formation. For some recalcitrant species or certain stages of development, media are sometimes supplemented with additional vitamins such as riboflavin, biotin, folic acid, and

pantothenic acid. Such vitamin requirements are species- and tissue-specific, and while a few cultures are even entirely capable of such vitamin autotrophy, others display stricter requirements for exogenous vitamin provision. This variability makes it essential to empirically determine the optimal concentrations of vitamins for individual culture systems.

Carbohydrates

In tissue culture media, carbohydrates play are an energy source and osmotic regulators. Exogenous sugars supply the required carbon and energy for growth and development because most cultures function by heterotrophic or mixotrophic conditions with restricted or no photosynthetic ability. In most media formulations, sucrose is the carbohydrate of choice and this is most typically at a concentration of 2-5%, and more commonly at 3% (30 g/L). When some metabolic pathway ought to be manipulated, glucose and fructose can occasionally be used in lieu of sucrose, but they are usually less effective in respect of a greater part of uses. Specialized culture systems, such as those designed for embryogenesis protocols or osmotic adjustment, often employ alternative carbohydrates, such as maltose, lactose and sorbitol. Carbohydrate content has a major impact on culture morphogenesis high levels can enhance somatic embryogenesis, or suppress organogenesis, depending on the species. Moreover, high sugar concentrations can induce osmotic stress while low concentrations constrain growth as energy is not freely available. Consequently, one must carefully optimize the carbohydrate component dependent on culture goals and species traits.

Growth Regulators

Plant growth regulators (PGRs) induce morphogenetic responses in tissue cultures, determining the balance between cell division, expansion and differentiation. These potent signaling molecules operate at remarkably low concentrations, often in the micromolar or nanomolar range, but have a considerable impact on developmental trajectories. Auxins, cytokinins, gibberellins, abscisic acid and ethylene are the five primary classes of common plant growth regulators that play various roles during culture development. Auxins, whether the natural forms indole-3-acetic acid (IAA)

or synthetic analogs such as 2,4-dichlorophenoxyacetic acid (2,4-D), naphthaleneacetic acid (NAA), and indole-3-butyric acid (IBA), are primarily responsible for cell division and elongation and root formation. Their media concentration has a considerable impact on morphogenetic patterns, where a high auxin concentration usually promotes callus formation and encourages root growth and inhibits bud growth. However, while the synthetic auxin 2,4-D is particularly potent in inducing callus and embryogenic cultures, it may inhibit regeneration later in the culture process unless it is removed or reduced (Bhaskar et al. 2010, Ishikawa et al. 2011). Cytokinins, such as the naturally occurring zeatin and synthetics such as 6-benzylaminopurine (BAP), kinetin, and thidiazuron (TDZ) act as auxin antagonists and promote cell division, overcome apical dominance and induce shoot formation. The morphogenetic fate is often determined by auxin-to-cytokinin ratios, with high cytokinin-to-auxin ratios promoting shoot development and inverse ratios favoring rooting. Thidiazuron, a technical urea derivative that has cytokinin-like activity, shows extraordinary potency for woody species that are recalcitrant to conventional cytokinins. They are primarily represented in tissue culture applications by gibberellic acid (GA*f*) and are associated with cell elongation, breaking dormancy, andimproving germination. Their application is still more limited than auxins and cytokinins for tissue cultures, only used in particular cases, such as dormancy inhibition in seeds or for elongating microshoots. Abscisic acid (ABA) is involved in various aspects of plant life, including stress responses, dormancy induction, and embryo maturation, and can be used in somatic embryogenesis protocols to improve embryo quality and synchronize development. Ethylene produced by cultures themselves as a gaseous hormone usually inhibits in vitro growth and is therefore commonly added in recalcitrant culture systems as an inhibitor (e.g., silver thiosulfate or aminoethoxyvinylglycine). In some special applications, more growth regulators such as polyamines, brassinosteroids, jasmonates and salicylates are also used for more modern tissue culture. The exact regulation of growth regulator type, concentration, and timing may be one of the most important determinants regulating morphogenetic responses in tissue culture systems.

Gelling Agents

For solid and semi-solid media, gelling agents provide physical support for explants and modulate nutrient availability via their interaction with media components. Currently,

agar, a polysaccharide obtained from red algae, is the most used solidifying agent because it is stable, transparent and resistant to enzymatic degradation by the plant tissues. However, agar is rarely perfectly pure and the presence of various undesirable compounds can lead to worst performance of gas phase. While purified agar derivatives such as agarose have much greater clarity and lower impurity levels, they also come at a significant cost premium. Other gelling agents include Gellan gum (e.g., Gelrite, Phytagel) which produces clearer gels at lower concentrations than agar; Carrageenan, whose origin is in red seaweed; and synthetic polymers such as Polyacrylamide for different applications. The gel matrix physical properties strongly affect water potential, gas exchange and nutrients availability. Higher concentration equates to less uptake of radicals compared to lower concentration, however lower concentrations allow for better biomass, anti-microbial flow as well as a softer more cohesive gel that may facilitate the absorption of the explant, ie, a softer gel may work more efficiently in absorbing nutrients than a harder one. Even more, the interaction between gelling agents and media components, such as divalent cations (i.e. calcium and magnesium), presents unique challenges to media design and requires specific empirical optimization for a particular application.

pH and Buffers

Within the tissue culture media, the pH is critical with regards to nutrient solubility, enzyme activity, and cellular uptake systems. The majority of the plant tissue culture media have a pH range before autoclacing between 5.4 and 5.8 that usually falls 0.1-0.3 units after sterilization. This mildly acidic range allows for optimal available mineral nutrients and supports cellular functions. pH adjustment in media preparation with dilute acids (HCl) or bases (NaOH or KOH) is commonly done before sterilization. Buffer presence can also be beneficial for the longer culture periods as the media gets more basic type from ion exchange process and the plant's metabolic activities. Examples of buffer agents: MES (2-(N-morpholino) ethanesulfonic acid) (for the pH range 5.5-6.5) and HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (for the pH range 6.8-8.2). These buffers demonstrate an ability to stabilize the pH of the media throughout the culture cycle, which is especially critical to sensitive species or prolonged culture times. But adding them will add to media cost and potentially interact

with other media components, so careful consideration will be needed as to whether the benefits of adding them outweigh the potential downsides.

Antioxidants and Adsorbents

During explant preparation, many plant tissues, especially those of woody species, exude phenolic compounds and other oxidative agents when damaged. Those compounds polymerize into dark, toxic products that inhibit growth and cause tissue browning and eventual death. Antioxidants counter these effects, either by preventing oxidation or by absorbing harmful compounds. Provided tissue culture media may contain different common antioxidants such as ascorbic acid, citric acid, polyvinylpyrrolidone (PVP), activated charcoal, cysteine and glutathione. The two ascorbic acid and citric acid are reducing agents and PVP and activated charcoal absorb the phenolic compounds, thus not allowing them to be oxidized. These factors are especially useful during culture initiation from woody, phenolic-rich explants. Activated charcoal is especially worth mentioning for its multifunctional role in tissue culture. In addition to adsorbing toxic compounds, it has been shown to bind growth regulators, decreasing the effective concentration of these components in media. This property may be exploited to gradually lower growth regulator levels during the culture process without the need for media exchanges. New benefits of activated charcoal are that it creates a darkened environment, this helps to simulate soil conditions that could assist with root growth and somatic embryogenesis in some species.

Antimicrobials and Antibiotics

Contamination of cultures by microorganisms has proved to be a chronic problem for plant tissue culture, even following the most stringent aseptic techniques. Occasionally, antibiotics such as streptomycin, gentamicin, cefotaxime, and kanamycin are added to media to prevent bacterial contamination. Fungal contamination problems are handled by fungicides like benomyl, carbendazim, and nystatin. However, these compounds may be phytotoxic themselves, preventing plant growth at concentrations that are effective against microbial contaminants. In addition, the introduction of such vectors may aid in the selection of resistant microbial strains or affect certain aspects of plant regeneration pathways. Natural antimicrobial products such as plant essential oils derived from thyme, oregano and cinnamon have recently attracted interest as less

phytotoxic alternatives to conventional antibiotics. These natural antimicrobials might also have a broader spectrum of activity against bacterial and fungal contaminants with lower toxicity to plant tissues. However, the best strategy for contaminant control is prevention through proper aseptic technique, not the use of antimicrobial additives.

Natural Complex Additives

Undefined natural extracts commonly enrich defined media constituents to improve growth performance. Coconut water is a widely used growth and development hormone source in culture systems and is known to stimulate cell division and embryo development, especially as it is rich in cytokinins, amino acids, vitamins, and minerals. Showing growth for recalcitrant speciesCasein hydrolysate is a mixture of amino acids and small peptides which stimulate growth. Yeast extract provides vitamins, especially all the B complex and amino acids in addition to other growth factors. Malt Extract Provides Carbohydrates, Proteins and Hormones Useful to some types of culture. Other natural supplements that have been used for certain purposes are banana homogenate, tomato juice, orange juice, and potato extract. Although these complex additives improve culture performance in general, their undefined and variable composition involves inconsistency among batches and a nightmare of reproducibility across experimental paradigms. In contemporary tissue culture, media formulations are increasingly defined, dispensing with complex additives in favour of identified active components wherever possible. However, these natural supplements remain critical for recalcitrant species for which defined media formulations have not yet been successful.

Types of Tissue Culture Media

Classification schemes for tissue culture media include application-based, chemical composition-based, physical state-based and researcher-based classification. The criteria of each classification provide different views on the features and uses of media and that allows researchers to choose the suitable formulations for their own goals.

Chemical Composition based Classification

Inorganic Media

Initial attempts in the culture of tissue were based on comparatively mild solutions of inorganic salts, which were augmented by suspension of a carbohydrate. An example of this solution is Knop's solution (1865) which was developed for plant growth in solution (i.e., hydroponics) and contains a basic formulation of calcium nitrate, potassium nitrate, magnesium sulfate, potassium phosphate, and iron chloride. These basic formulations supported limited outgrowth of some robust tissue types, but were inadequate for maintaining most plant tissues (need>micronutrients, organic compounds (cellular differentiation, morphogenesis).

Synthetic or Defined Media

Synthetic or defined media with well-defined chemical constituents at prescribed concentrations are used for modern tissue culture. These formulations allow for reproducibility of the experimental conditions, as well as systematic optimization via the modification of individual components. Some defined media include Murashige and Skoog (MS) medium, Gamborg's B5, Nitsch and Nitsch medium and White's medium: they all contain well-defined concentrations of macronutrients, micronutrients, vitamins and other organic supplements. Furthermore, the well-defined composition of these media enables researchers to systematically assess nutritional requirements and to formulate specialized media for certain species or tissue types.

Natural or Complex Media

Although defined media offers many benefits, some recalcitrant species or specialized applications can benefit from natural extracts that possess complex, undefined compositions. This type includes media that contain potato homogeneate or banana pulp and also coconut water, yeast extract, or malt extract. They are not discretely defined chemically, yet these natural additives offer growth factors, hormones, vitamins, and amino acids that are useful for difficult culture systems. The main disadvantage is the loss of experimental reproducibility due to batch-to-batch variability, leading to a modern-day preference for defined alternatives where possible.

Physical State Based Classification

Liquid Media

Liquid media do not contain gelling agents, allowing for total immersion of the tissue and maximizing the surface area for contact with nutrients. This format is especially beneficial for suspension cultures, protoplast cultures, microspore cultures and somatic embryogenesis systems in which rapid cell division and optimal nutrient availability takes precedence. Here, we present an in vivo crowdfunding approach where synthetic transgenic tissues can be assembled to establish automatically perfused organoids, using a liquid media system that allows for improved growth rates and decreased nutrient diffusion and tissue asphyxiation through filter paper bridges, mesh support, or shaking/rotation mechanisms. This lack of physical support can result in the development of tissue hyperhydricity (previously referred to as vitrification), a physiological disorder characterized by water-soaked, translucent tissues with abnormal morphology and decreased regenerative capacity (see text Box 1).

Semi-solid Media

Semi-solid or gelled media includes solidifying agents (default 0.6-0.8% agar) which contribute to a gel matrix that physically holds explants while providing the free movement of nutrients within the medium. This is the most frequent physical state in the context of conventional tissue culture, which is providing enough support and reasonable access to nutrients. This solid surface provides the appropriate orientation of the explants and also promotes proper polarized development that is essential for organogenesis and embryogenesis. Gel concentration affects culture performance, as higher gelling concentrations provide improved mechanical support but may hinder nutrient availability and diffusion, while lower gelling concentrations improve nutrient access but result in tissue submerging.

Double-phase Media

This led to the development of double-phase systems, which try to benefit from the advantages of both formats by having two phases (liquid and solid) in the same culture vessel. Very commonly, a layer of liquid medium is placed over a gelled base, allowing the explants to maintain contact with a solid support while still being able to access the nutrients from the liquid phase. This is especially useful for those cultures that can benefit from being exposed to varying media compositions on a step-wise basis, or where hyperhydricity is a concern but can be controlled while at high levels of nutrients

available. A significant variant is the thin-layer liquid culture, which allows a reduced volume of liquid medium to be present on a solid support matrix and combines the advantages of both systems while mitigating the hyperhydricity risks.

Application or Purpose Based Classification

Media for Isolation or Primary Culture

Establishment of initial culture from field-grown or greenhouse plants poses specific challenges, namely, control of contamination and browning of the explants, due to the action of phenolic oxydases. Isolation media usually contains elevated concentrations of plant preservative mixture (PPM), antioxidants including ascorbic acid or citric acid, adsorbents such as activated charcoal or polyvinylpyrrolidone (PVP), and might employ stronger sterilants for sterilization. These have specialized formulations designed to promote explant survival in the critical establishment phase, but not necessarily optimizing growth or morphogenesis, which are of greatest interest for growth/ secondary culture stages.

Multiplying or Proliferating Media

After cultures successfully establish, multiplication media are employed to induce rapid growth and proliferation with even higher levels of cytokinin (usually BAP or TDZ) compared to auxins. These media formulations generally also have optimized macro and micronutrient concentrations supporting the sustained cell division and the organogenesis. Cytokinin-to-auxin ratios that promote shoot initiation are beneficial for shoot multiplication whereas callus multiplication likely operates using balanced ratios promoting undifferentiated growth. Multiplication media generally also have a higher level of nitrogen and may also contain organic nitrogen sources, such as glutamine or casein hydrolysate, which stimulate accelerated protein synthesis during periods of rapid growth.

Rooting Media

Root induction often necessitates different nutritional and hormonal milieu than that which supports shoot multiplication. Rooting media typically consist of a half-strength MS nutrients (or quarter strength MS salts) with no cytokinins or with reduced or

absent cytokinins, and supplemented with auxins (especially IBA or NAA) for the induction of rhizogenesis. Certain formulations utilize activated charcoal to adsorb upon inhibitory compounds and provide a darkened environment that mimics soil conditions. Furthermore, rooting media could lessen or supplant ammonium nitrogen with nitrate forms, since so much of ammonium in the rooting media has been shown to suppress the root formation of many species.

Shooting Media

Induced shoot media promote axillary bud outgrowth or adventitious shoot formation by enhanced cytokinin concentrations usually accompanied by reduced auxin levels. These formulations preserve full-strength macronutrients that support active metabolism and morphogenesis, with nitrogen sources tailored to protein synthesis and structural advancement. The particular type of cytokinin matters, with benzyladenine (BAP), for example, being permissive of axillary shoot development, while thidiazuron (TDZ) can be permissive of both axillary and adventitious shoot types, especially in woody type species.

Basal Media

Basal media typically assume a supporting role, providing basic nutrition without adding growth regulators, which allow them the potential to serve as the bases for specialized media by augmenting with hormones. These derivatives provide essential macro and micronutrients, vitamins, carbohydrates, and other organic compounds necessary for basal cellular maintenance. Basal media, such as MS, B5, and WPM are commonly used for developing media for various applications by supplementing or adding desired growth regulators and nutrients. This modular setup allows for media optimization in a systematic way across stages of culture, while within the same experiment.

Formulations of Flagship Established Media

Murashige and Skoog (MS) Medium

The full name is the Murashige–Skoog medium, and it was formulated in 1962 by Toshio Murashige and Folke Skoog when classifying the growth requirements of tobacco tissue cultures. MS medium is the most utilized formulation in plant tissue

culture applications. Other prominent features are moderate to relatively high nitrogen and potassium contents, with nitrogen supplied in a ratio of about 2:1 as nitrate and ammonium. It includes macronutrients (nitrogen, phosphorus, potassium, calcium, magnesium, and sulfur), micronutrients (iron, manganese, zinc, boron, copper, molybdenum, cobalt, and iodine), organic components (myo-inositol, nicotinic acid, pyridoxine HCl, thiamine HCl, and glycine), and usually 3% sucrose as a carbon source. The success of MS medium can be attributed to its nutritious and balanced composition supporting a wide range of plant species and tissue types. Partial strengths such as ½MS for rooting, germination, etc. and media with varying nitrogen sources, vitamin contents, or micronutrient concentrations for different species needs are also available. The MS medium still serves as the basic medium for most tissue culture applications after 60 years, attesting for its extraordinary proven versatility across the plant phyla.

Gamborg's B5 Medium

Based on a study by Oluf Gamborg and others in 1968, B5 medium was developed for soybean callus and cell suspension cultures. B5 has lower nitrogen levels and a higher nitrate-to-ammonium ratio compared to MS (Gibson et al. 2006), leading to less ammonium toxicity in sensitive species. The medium has a lower overall salt concentration, but sufficiently provides trace elements and has a unique vitamin mix with higher levels of thiamine. This enhances the adaptability of B5 to legumes, many woody species, and cell suspension cultures in which decreased ammonium concentrations are advantageous. The factors of B5 medium usually outperform MS for protoplast cultures, certain species of woody plants, and cultures that are low salt stress. Its widespread use for leguminous plants, such as soybean, originates from the development of this system with soybean systems. Media for specific applications may consist of part MS and part B5 formulation, as researchers often create hybrid formulations based on properties from both parent media to optimize for a specific application.

Woody Plant Medium (WPM)

Woody Plant Medium was developed by Lloyd and McCown in 1980 to meet the nutritional needs of recalcitrant woody species that do not grow well on conventional media. Compared to MS, WPM has a much lower salt concentration, due to low levels of ammonium and nitrate, but relatively balanced levels of calcium and magnesium. Instead of chlorides as counterions for multiple minerals, the formulation uses sulfates, which might conceivably be less toxic to sensitive woody species. These attributes make WPM very well suited for woody ornamentals, forest tree species, and fruit crops that may perform poorly on higher-salt media such as MS. The low ionic strength of WPM alleviates osmotic stress and its unique micronutrient composition is tailored for specific woody needs. This specialized culture medium has allowed numerous economically important woody species that were once considered recalcitrant to tissue culture including many from the Rosaceae, Ericaceae, and Pinaceae families to be successfully micropropagated.

Nitsch and Nitsch Medium (NN)

Nitsch and Nitsch medium, developed for anther culture and microspore embryogenesis, has relatively low nitrogen content as compared to MS and biotin and folic acid are included in its vitamin mixture. This formulation affords a balanced nutrition system to mediate androgenesis (development of haploid embryos from male gametophytic cells), while limiting osmotic and salt stress that could restrict gametophytic to sporophytic transition. Many media supporting haploid production in various plant families are based on NN medium.

White's Medium

One of the first defined tissue culture formulations was White's medium, developed by Philip R. White in the 1930s for root culture. It has relatively lower concentrations of salt, especially nitrogen, and is deficient in some micronutrients by modern standards. I often find White's medium used less frequently due to these 2 downsides however, it sees great utility with root cultures, orchid seed germination, and certain sensitive species in need of less salt exposure. Leaving historical significance aside, contemporary uses of White's formula usually include adaptations that remedy its nutritional inadequacies but maintain its mild, low-salt profile.

Vacin and Went Medium (VW)

VW medium is formulated with lower levels of nitrogen and includes some complex additives, to suit orchid culture. This formula is based on the special nutritional needs of orchids (many are epiphytes and have adapted to nutrient-poor environments). Despite the availability of newer orchid-specific formulations, VW and its derivatives are still widely used for orchid seed germination, protocorm development and micropropagation.

DKW Medium

Driver and Kuniyuki created DKW medium specifically for walnut (Juglans) micropropagation, as the nutritional needs of this difficult woody genus are quite specific. The formulation contains elevated calcium and magnesium compared with MS, and altered micronutrient composition with added nickel. These traits have been advantageous not just for walnut but also to other nut crops and by all means other woody species in the Juglandaceae family as well. These specialized media are required to fulfill a growing need owing to numerous economically significant but culturally difficult groups of plants, and DKW is a case in point in this continuing evolution.

Advancements in Tissue Culture Media

Advancements in tissue culture media are ongoing through innovations in technology, increased understanding of plant physiology, and experience through practical application. Recent technologies emphasize improving culture efficiency, lowering costs, overcoming recalcitrance in difficult species, and devising sustainable solutions in harmony with wider environmental concerns. To address the obstacles presented by traditional complex additives, modern tissue culture is moving towards chemically defined media, marking and isolating specific active compounds and using those in its applications. This change facilitates reproducibility of experiments and allows manipulation of nutrition in a highly controlled manner. For instance, certain cytokinins, amino acids, and inositol phosphates have been isolated from coconut water; they have been found to replace the complex extract in many applications. Similarly, individual peptides from a casein hydrolysate and defined vitamin mixtures in place of yeast extract have also permitted the formulation of completely defined media for previously

recalcitrant species. Such identification of bioactive compounds in complex extracts can be better achieved with the use of advanced analytical methods such as high-performance liquid chromatography (HPLC), mass spectrometry, and nuclear magnetic resonance (NMR) spectroscopy. Once identified, these compounds can then be synthesized or isolated in pure form and incorporated at a certain concentration. This method retains many advantages of complex additives yet avoids the inconsistencies and ambiguity associated with such approaches that are a bioprocessing paradigm shift.

Synthetic Seed Technology

The novel synthetic or artificial seed technology involves capsule of somatic embryos or micropropagated propagules in gel matrices (usually alginate) containing nutrients, growth regulators and protective compounds. This innovation produces naturally mimetic, seed-like handling properties propagules that carry clonal, elite germplasm. This encapsulation matrix contains traditional media components as well as osmoprotectants, antimicrobials and slow-release nutrients to allow for effective propagule survival in storage and immediate onset of growth post-planting. Recent developments in synthetic seed technology have added beneficial microorganisms into the polymer-based encapsulates, embedding mycorrhizal fungi or plant growth-promoting rhizobacteria in the encapsulation matrix, resulting in "enhanced" synthetic seeds that exhibit better establishment characteristics. A promising frontier is the development of desiccation-tolerant synthetic seeds with the addition of abscisic acid, trehalose, and other protective compounds during the encapsulation process, which, in the absence of previous interventions, could lead to storage of recalcitrant germplasm at ambient temperature. These advances link bench-based tissue culture to agro-environmental deployment allowing for technology transfer to agriculture.

Bioreactors Accumulation and Liquid Culture Biology

The use of bioreactor technology has been a game changer in large-scale tissue culture via liquid culture in passive (manual) environmental control. These systems maximize gas exchange, nutrient delivery, and physical stress factors while minimizing the labor costs associated with conventional methods. They are temporary immersion systems, such as RITA® (Recipient for Automated Temporary Immersion) and TIB (Temporary

Immersion Bioreactor), periodically immersing with a liquid medium, allowing to take the advantages of liquid media while decreasing hyperhydricity risk. Such systems are especially useful for somatic embryogenesis, bulblet production, and microtuber formation in a wide array of species. State-of-the-art bioreactor designs are capable of continuously monitoring culture parameters such as pH, dissolved oxygen, sugar concentration, and mineral uptake, facilitating dynamic media adjustments during the culture cycle. More complex systems utilise AI algorithms to deliver the constituent nutrients in the optimal minutes / hours as they observe the culture growing via their computer vision systems. Media formulations engineered specifically for bioreactor applications typically incorporate antifoaming agents, optimized aeration components, and altered micronutrient delivery systems to account for unique physical dynamics in liquid culture spaces. Somatic embryogenesis, or the development of embryo-like structures from somatic cells, is a powerful regeneration pathway which necessitates specific media formulations. Recent developments target accurate modulation of the transition between phases of embryogenic induction, embryo development, maturation, and germination by using stage specific media compositions. Induction media are rich in auxin (especially 2,4-D) and specific nitrogen sources and the presence of osmotic agents such as polyethylene glycol (PEG). Development media deplete auxins and add certain signal molecules, including abscisic acid (ABA), brassinosteroids and jasmonic acid (JA) derivatives that improve embryo quality and synchronize embryogenesis . Somatic embryo maturation media increasingly incorporate compounds that replicate processes of natural seed maturation, such as desiccation under controlled conditions, dormancy-breaking treatments and embryo-specific nutrients facilitating the accumulation of reserves. The advances have enabled somatic embryogenesis application in previously recalcitrant species, in particular those of conifers and woody angiosperms with high economic relevance. These validated media sequences yield knowledge about basic embryological phases but may also be applied in a commercial propagation setting.

Media Optimisation Specific To a Species

Realizing that widely-used media formulations often fall short in fulfilling species-specific needs, contemporary tissue culture gradually acknowledges a growing need of plant specific formulation of tissue culture media. This trend uses systematic methods such

as factorial design experiments, response surface methodology, and artificial neural networks to screen multiple variables in a parallelized fashion, identifying optimal media compositions more efficiently. As a result, the developed more species-specific media most of the times show a substantial increase in regeneration efficiency, decreased culture time, and increased economic viability of micropropagation systems. They have been notable forms like media for recalcitrant crops such as cacao, coffee, and coconut; endangered species of concern for conservation; and commercial ornamentals with specific interest. Such formulations typically modify ratios of specific macronutrients, add specific organic additives to the mixture, or optimize micronutrient matrices to address unique metabolic features of specific plants. As taxonomic patterns in nutritional needs slowly become clear from these optimization studies, prediction of effective media compositions for species that remain uncultivated is likely to be informed by their phylogenetic relationships to well-studied taxa.

Nanomaterials for Additives and Micronutrient Delivery

The use of nanotechnology in tissue culture media is an emerging frontier that holds considerable potential benefits. Nanoparticles of basic components such as zinc, iron, and silver increase bioavailability and decrease toxicity in contrast to traditional salt forms. These nanoformulations can offer sustained nutrient release and minimize precipitation and interaction with the other media components. Zinc oxide nanoparticles promote growth and have antimicrobial effects in several types of culture systems, while iron nanoparticles show increased availability, not requiring chelating agents such as EDTA, which has other effects on culture development. In addition to direct nutritional applications, functionalized nanoparticles allow the delivery of growth regulators, genetic material, or even antimicrobial compounds to specific cell types in culture systems. In specific systems, carbon nanotubes and graphene-based materials may work as growth enhancers and gene delivery agents, however, further research is needed to ascertain their long-term safety and environmental impacts. However, these new applications may hold great promise in overcoming long-standing challenges associated with tissue culture media formulation.

Eco-Friendly and Budget-Friendly Solutions

Environmental sustainability concerns and cost considerations lead to the development of eco-friendly alternatives to traditional media components. Conventional gelling agents such as agar obtained from more endangered marine algae species are being supplanted by sustainable substitutes such as starches, pectins, and synthetic hydrogels with lower environmental impact. Likewise, scientists are looking at replacing expensive purified sugars with agricultural byproducts such as sugarcane molasses, corn steep liquor, or wastes from fruit processing, that provide carbohydrates at a lower cost and address industrial waste streams. Water conservation is another area of sustainability focus, including development of culture systems that use reduced volume, water recycling protocols and media formulations that do not require subculturing as frequently. Such systems not only reduce water use but also energy costs for sterilization. The general trend toward "greener" tissue culture is also in line with wider scientific focus on sustainable practice but also serves to potentially broaden accessibility to the tech by decreasing input curve.

Specialized media

Generation of Cryopreservation Pre-Treatment Media

Cryopreservation (the storage of plant germplasm at ultra-low temperatures) requires specialized preconditioning media that prepare tissues for freezing stress. These formulations usually include osmotic agents such as sucrose, mannitol, or sorbitol at high concentrations and cryoprotectants such as dimethyl sulfoxide (DMSO), glycerol, or proline. Newest advances are antioxidant containing media like glutathione, ascorbic acid and tocopherol that decrease oxidative damage from freezing and thawing. Other innovations include adding membrane stabilizers such as trehalose and raffinose, and controlled exposure to mild stress conditions inducing physiological adaptive responses which improve post-thaw recovery.

Protoplast Culture Media

Protoplasts (plant cells with cell walls enzymatically depleted) necessitate a highly specialized media based on their fragility and peculiarity of nutritional needs.» Modern-day protoplast medium includes osmotic stabilizers such as mannitol or sorbitol which prevent cell lysis, nurse cultures or conditioned media that supply growth factors, and

precisely adjusted calcium concentrations which help with membrane stability. Recently, reports have identified specific peptides, arabinogalactan proteins and chitosan derivatives that can promote cell wall formation and subsequent division in protoplast-derived cells. These advances have increased protoplast technology applications in previously recalcitrant species, thus augmenting their utilities for somatic hybridization and genetic transformation applications.

Evolution and Renewal Media

Protocol for genetic transformation relies on specific media supporting both transformation process itself and regeneration of transformative cells thereafter. These formulations combine the toxicity of the selection agent (antibiotics or herbicides) with nutritional support for the otherwise stressed transformed cells. Recent progress has included a two-phase media that separates the transformation and regeneration phases such that the initial media is optimized for Agrobacterium exposure or direct uptake of DNA, and second through experience with clones with enhanced growth (e.g., growth factor-optimized) after recovery through selection.

Unit 03: Growth Regulators

Plant growth regulators (PGRs) are natural or synthetic plant hormones that influence plant growth and development even at low concentrations. These chemical messengers orchestrate complex developmental processes and environmental responses over a plant's life cycle. In contrast to nutrients, which are directly incorporated into the development of plant tissues, PGRs act as signaling molecules to the plant that must then initiate specific physiological responses via a series of complex biochemical pathways. PGR discovery started in the early 20th century, first with auxins, then gibberellins, cytokinins, abscisic acid, and, finally, ethylene. These five main classes are the classical plant hormones. Over the last few decades researchers have isolated additional signaling compounds (that control plant growth), including brassinosteroids, jasmonates, salicylates, strigolactones, and peptide hormones. PGRs are widely used in modern agriculture and horticulture to control and influence plant growth, development, and responses to stresses. Such applications secure return on investment (ROI), improved yield, quality, and production efficiency in operation within various cropping systems. The strategic application of PGRs is a cutting-edge method of

crop management that enhances long-standing methods of fertilization, irrigation, and pest management.

Types of Plant Growth Regulators (PGRs)

Auxins

The first plant hormones to be discovered were auxins, predominantly indole-3-acetic acid (IAA). The groundwork for auxin discovery was laid by the initial observation of phototropism by Charles Darwin and his son, Francis, in canary grass seedlings. In 1926 Frits Warmolt Went isolated the active ingredient, which he labelled auxin, from Greek auxein meaning to grow. True auxins (mostly IAA) are produced in the meristematic regions of the plant: shoot apices; young leaves and growing seeds. IAA consists of the indole ring obtained from tryptophan, providing the core structure required for IAA. Synthetic auxins, such as 2,4-dichlorophenoxyacetic acid (2,4-D), naphthaleneacetic acid (NAA), and indolebutyric acid (IBA), mimic the effects of the native auxin but are often more stable in the environment. Auxins regulate a wide range of physiological functions from regulating cell elongation, apical dominance, vascular differentiation, root initiation, and many other processes. They promote cell enlargement by inducing proton pumps that acidify cell walls, allowing expansin proteins to relax cellulose microfibrils. This mechanism, termed acid growth, enhances the plasticity of cell walls and promotes water uptake, leading to cell expansion. In apical dominance, auxins in basipetal transport from shoot apex inhibit growth of the lateral buds. This mechanism ensures that vertical growth is prioritized before divergence occurs. The auxin gradient dissipates when the shoot apex is removed, resulting in lateral buds being released from inhibition and branching. Auxins are also involved in the growth of plants towards or away from environmental stimuli known as tropisms. In phototropism, light induces asymmetrical distribution of the plant hormone auxin, where the shaded side has a higher concentration of auxin that accelerates differential cell elongation, resulting in the bending of the stem towards light. In a similar plant tropism, gravitropism refers to the redistribution of auxins in response to gravity, causing root growth downwards and shoot growth upwards.

Gibberellins

Gibberellins (GAs) constitute a large family of tetracyclic diterpenoid carboxylic acids with more than 130 forms discovered in plants, fungi and bacteria. These compounds were first characterized from Gibberella fujikuroi, a fungus that causes "bakanae" or "foolish seedling" disease on rice. The active compound was isolated in 1926 by Japanese scientist Eiichi Kurosawa, with its chemical structure later established in the 1950s. GA1, GA3, GA4 and GA7 are biologically active forms in plants. GA is synthesized via the terpenoid pathway, beginning with geranylgeranyl diphosphate (GGPP) and via a series of complex oxidation reactions catalyzed by terpene synthases, cytochrome P450 monooxygenases and 2-oxoglutarate-dependent dioxygenases. The major effect of gibberellins is to promote stem elongation by: (a) increasing cell division; and (b) increasing cell elongation. In contrast to auxins, which primarily regulate cell expansion, GAs stimulate both cell number and cell size. In genetic dwarfs and rosette plants, this effect is even more dramatic, and GA application can restore growth to normal levels. One of the defining functions of gibberellins is during the germination of seeds. In the aleurone layer of the cereal grain, they induce production and release of hydrolytic enzymes (especially á-amylase). These enzymes mobilize endosperm reserves supplying energy and nutrients to the developing embryo. Seeds deficient in GA commonly stay dormant, while treatment with GA can initiate germination by breaking dormancy (10) and, is characterized by the emergence of radicle as the first germination criterion (15). Gibberellins are also involved in reproductive development, influencing processes like flower induction, sex expression and fruit development. They can act as a substitute for cold requirements (vernalization) in particular species of biennial plants, allowing them to flower without cold exposure. In a plethora of species, GAs are shown to promote male flowers formation, whilst the formation of female flowers is inhibited, which shows the role of floral sex determination.

Cytokinins

Cytokinins are derivatives of adenine at N6 position with a side chain. They were first identified by Folke Skoog and Carlos Miller in 1955 while looking for compounds that stimulate cell division in tobacco tissue cultures. The first naturally occurring cytokinin to be isolated was zeatin from corn (Zea mays) endosperm, for which it was named. Natural cytokinins are zeatin, dihydrozeatin, and isopentenyladenine and occur

as free bases, ribosides, or ribotides. Synthetic cytokinins such as kinetin, benzyladenine (BA), and thidiazuron (TDZ) often show more stability and activity than their natural homologs. The biosynthesis of cytokinin takes place mainly in root tips, developing embryos and actively dividing tissues. Isopentenyl transferase (IPT) is a rate-limiting enzyme that catalyzes the transfer of an isopentanyl moiety from dimethylallyl pyrophosphate to adenosine phosphates. Primarily, transport is through the xylem, transporting root-derived cytokinins to the shoots. Cytokinins are fundamental in promoting cell division, mediated through regulation of the cell cycle. They accompany auxins in regulating morphogenesis in tissue culture by promoting G1-S and G2-M transitions in mitosis. The ratio of auxin to cytokinin influences cell differentiation, where increased auxin concentrations favour rooting, elevated cytokinin levels promote shoot differentiation and intermediate auxin and cytokinin concentrations yield un-differentiated growth as callus. Cytokinins suppress senescence by stabilizing chlorophyll, sustaining protein biosynthesis, and avoiding nutrient remobilization from senescing tissues. Cytokinin's "window effect" is something that can be seen with the naked eye when cytokinin is applied to a leaf spot, making it possible to see a green island in the middle of tissue yellowing around it. The other role of these hormones is apical dominance, they counteract auxin at the lateral bud and so decrease its effect (inhibits lateral bud development). Auxins originating from the shoot apex inhibit lateral bud growth, while root-generated cytokinins promote bud outgrowth. And this antagonistic relationship contributes to overall plant architecture regulation.

Ethylene

Ethylene (C_2H_4) is distinctive among plant hormones as a small gaseous hydrocarbon. The first noticeable effects on plants were noted in the late 1800s, when trees around street lamps fueled by illuminating gas defoliated as a result of exposure. In 1901, Dimitry Neljubow discovered the active component to be ethylene. Later, Russian scientist Nikolai Gimalayskiy showed that ripening fruits produce a substance that impels the ripening of nearby unripe fruits a substance that based on the 1925 work by Kidd and West was confirmed as being the gas ethylene. Ethylene The Simple Plant Hormone Unlike other plant hormones with complex structures, ethylene is a simple unsaturated hydrocarbon with a carbon-carbon double bond. Its pulmonary nature means it can diffuse easily through tissues and atmosphere, allowing them to

respond quickly and negotiate with neighboring plants. Ethylene is synthesized from the amino acid methionine in a three-step pathway. Methionine is converted into S-adenosylmethionine (SAM), which is then converted into 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase. And finally ACC oxidase acts on ACC to form ethylene, a reaction for which carbon dioxide and hydrogen cyanide are a byproduct. Ethylene production is upregulated during distinct developmental stages, including germination, flowering, fruit ripening, and senescence. Different stresses wounding, flooding, drought, and pathogen attack induce ethylene biosynthesis for plant defence responses. One of the most noticeable functions of ethylene is in the ripening of fruits, especially the climacteric fruits, such as that of apple, banana, and tomato. Ripening involves the action of ethylene which induces a series of biochemical alterations namely for fruit softening (due to the activity of cell wall degrading enzymes), color change (by chlorophyll degradation and carotenoid/anthocyanin production), sweetening (through starch hydrolysis) and aroma generation (via volatile compound formation). Ethylene also plays a role in the response of seedlings to mechanical barriers, an adaptive mechanism known as the triple response. In the presence of an obstacle, more ethylene is produced which causes (1) decreased stem elongation, (2) stem thickening, and (3) horizontal growth. This enables seedlings to circumvent soil obstructions during emergence. Under flooding condition, ethylene accumulates in hypoxic tissue and activates adaptive responses such as aerenchyma (air space in roots and stems), adventitious root (AR) development, and leaf epinastic (downward bending). These adaptations facilitate oxygen access and allow plants to survive waterlogging.

Abscisic Acid

Abscisic acid (ABA) was independently discovered by two research groups in 1960s. One team that studied the abscission of cotton bolls identified "abscisin II" and another, which focused on the bud dormancy of woody species, discovered "dormin". The compounds turned out to be the same and were named abscisic acid. ABA is a sesquiterpene with 15 carbons and contains a cyclohexane ring with an unsaturated side chain and terminal carboxylic acid group. There are cis and trans isomers, but only the (+)-cis-ABA has significant biological activity. Carotenoid pathway leads to ABA biosynthesis. During the process, this xanthophyll is cleaved by NCED (9-cis-

epoxycarotenoid dioxygenase) with the production of xanthoxin, which through oxidation steps becomes ABA. Drought and other environmental stresses induce NCED expression, consequently leading to a rapid increase of ABA levels. Commonly referred to as the "stress hormone," ABA regulates responses to drought, cold and salinity. When plants are under water deficit, the levels of ABA in leaves can increase 50-fold within a few hours, leading to stomatal closure to reduce transpirational water loss. This happens by a complex signaling cascade that involves the uptake of calcium and modulation of ion channels in guard cells which results in loss of turgor and closure of stomata. ABA is critical for seed maturation and dormancy (Finkelstein et al. 2008; Li et al. 2006). ABA stimulates the biosynthesis of storage proteins and late embryogenesis abundant (LEA) proteins that protect the embryonic tissues during desiccation during embryo development. ABA also induces dormancy by inhibiting germination until proper conditions arise, preventing precocious germination while the seed is still attached to the mother plant. During this stage of bud dormancy, ABA accumulates in response to shortening photoperiods in autumn and prepares woody perennials for overwintering. This dormancy protects cold-intolerant new growth from infection and colonization during transient warm spells in the winter. Both seed and bud dormancy have to be overcome by a period of moist chilling (stratification) or GA application, which is effective in overcoming ABA effects to resume growth.

Brassinosteroids

Brassinosteroids (BRs) are steroid hormones identified in 1979 from Brassica napus (rapeseed) pollen by Mitchell and coworkers. The most active BR was identified from a screen of 40 kg bee-collected pollen: brassinolide. Over 70 naturally occurring brassinosteroids have been characterized since. These molecules bear a close structural resemblance to animal steroid hormones, possessing a 5á-cholestan parent skeleton accompanied by hydroxyl and alkyl substituents. The most active structures include a seven-membered lactone of the B-ring. BR biosynthesis starts with campesterol and passes through a number of oxidation steps now known to be catalyzed by cytochrome P450s. Brassinosteroids stimulate cell elongation and division via different mechanisms than those of other plant hormones. They promote cell wall loosening for this purpose by inducing expansin genes and xyloglucan

endotransglycosylase, which precedes wall reinforcement through the induction of cellulose synthesis. This dual action promotes regulated expansion of the cells. BR-deficient mutants exhibit typical dwarf phenotypes, characterized by dark green and rounded leaves and lower fertility. In contrast to gibberellin-deficient dwarfs, BR mutants cannot be completely restored by applying the hormone exogenously late in development, suggesting key early roles that shape developmental trajectories. Brassinosteroids are involved in vascular formation by promoting xylem differentiation and inhibiting phloem formation. Furthermore they promote the formation of tracheary elements in zinnia elegans cell cultures, a model system which has been utilized for the study of xylogenesis. This role in vascular tissue development affects the plants body plan and vascular function. Brassinosteroids also improve stress tolerance, protecting against heat, cold, drought, and salinity. They induce antioxidant systems, gene expression related to stress-response, and osmoregulatory gene expression. This potent and broad spectrum stress protection has generated more interest in brassinosteroids for agricultural applications.

Jasmonates

Jasmonates are oxylipin-derived compounds originally identified in jasmine (Jasminum grandiflorum) essential oil. Jasmonic acid (JA) and derivatives, most notably methyl jasmonate (MeJA) and jasmonic acid isoleucine conjugate (JA-Ile), act as signaling molecules in response to stress and development. Jasmonate biosynthesis starts from membrane lipids, mainly á-linolenic acid released from chloroplast membranes by phospholipases. A series of enzymatic reactions are catalyzed by lipoxygenase, allene oxide synthase and allene oxide cyclase resulting in the production of 12-oxo-phytodienoic acid (OPDA) which gets reduced and then undergoes â-oxidation to generate JA (figure 1). Jasmonates are known mainly as wound and defense signal molecules. Mechanical damage or insect feeding quickly initiates synthesis of JA, leading to the expression of defensive genes such as proteinase inhibitors, polyphenol oxidases and other secondary metabolites that are toxic to herbivores. Plant-pathogen interactions are the complex signaling network in which jasmonates intercommunicate with salicylic acid. JA typically mediates resistance to necrotrophic pathogens and herbivorous insects, while salicylic acid governs defenses against biotrophic pathogens. Cross-talk between these pathways enables tailored responses to subsets of dangers.

Besides defense, jasmonates also modulate reproductive development, particularly stamen and pollen maturation. JA-deficient mutants are commonly male-sterile as a result of filament elongation defects, anther dehiscence, and defective pollen viability. Jasmonates are also used to up regulate the production of secondary metabolites, such as alkaloids, terpenoids, and phenylpropanoids, the many of which are of importance as medicinal and aromatic agents.

Salicylates

Salicylic acid (SA) is a phenolic compound named after Salix (willow) trees, which bark extracts containing salicylates have been used in medicine for centuries. Although previously mostly known as a precursor for an analgesic (related to aspirin), SA was recognized as a plant hormone with the discovery of its role in systemic acquired resistance. Structurally, SA is a benzene ring with hydroxyl and carboxyl in ortho position. Two separate biosynthetic pathways are responsible for the synthesis of SA in plants, one being the phenylalanine ammonia-lyase (PAL) pathway, which employs phenylalanine, and the other being the isochorismate synthase (ICS) pathway, which is based on chorismate in the shikimate pathway. During a pathogen challenge, the ICS route prevails. Salicylic acid acting as the main mediator of systemic acquired resistance (SAR), a form of broad-spectrum immunity induced following localized pathogen exposure. After initial infection, SA builds up both locally and systemically, triggering the expression of pathogenesis-related (PR) genes that encode antimicrobial proteins. This "immunization" defends distant tissues from subsequent assaults by pathogens. SA signaling is mediated by the regulatory protein NPR1 (NONEXPRESSOR OF PR GENES1). NPR1 is present as an inactive oligomer localized in the cytoplasm under basal conditions. SA mediated changes in cellular redox status resulted in the reduction of NPR1 into active monomers that translocate into the nucleus, where they interact with TGA transcription factors to upregulate defense gene expression. In addition to pathogen defense, SA also promotes thermogenesis and heat production within specialized plants such as skunk cabbage (Symplocarpus foetidus). By uncoupling respiratory electron transport from ATP synthesis, SA further augments metabolic heat generation, thus helping thermogenic flowers to melt their way through snow or attract pollinators. SA also impacts flowering time, especially under stress conditions, and induce alternative respiratory pathways.

Exogenous SA application generally acts as an inhibitor on seed germination, ethylene output, and fruit ripening, reflecting complex crosstalk between other hormonal systems.

Strigolactones

Strigolactones (SLs) are one of the latest discovered groups of plant hormones. Originally labelled as germination stimulators for parasitic weeds in the Striga genus, it was not until 2008 studies on branching mutants confirmed their identification as endogenous plant growth regulators. These compounds consist of a butenolide cycle (D-ring) linked to a tricyclic lactone (ABC rings) via an enol ether bridge. Strigolactone natural products include strigol, orobanchol and 5-deoxystrigol, with many structural variants being reported throughout plant species. The biosynthetic pathway of strigolactones begins with â-carotene and includes sequential modifications catalyzed by carotenoid cleavage dioxygenases (CCD7 and CCD8) and cytochrome P450 enzymes . Roots are the primary sites of production and synthesizes upon phosphate and nitrogen starvation. Strigolactones are best known for their ability to inhibit shoot branching. They function downstream of auxin in modulating axillary bud outgrowth, promoting apical dominance. Mutants with defects in strigolactone biosynthesis display bushy phenotypes characterized by tillering or branching, which confirm the role of the hormone in fine-tuning shoot growth depending on nutrient availability. In the rhizosphere, strogilactones act as a signaling molecule which stimulates parasite branching in arbuscular mycorrhizae, promoting symbiotic interactions to allow more efficient nutrient uptake, especially of phosphorus. This function probably existed before their function in regulating plant development, illustrating an evolutionary co-options of rhizosphere signaling for the regulation of internal growth. The paradox is that the same compounds that are beneficial to plants through mycorrhizal symbiosis can also have harmful effects by inducing germination of parasitic weeds such as Striga and Orobanche. Strigolactones have evolved as signaling substances for these parasites, indicating the (proximity of a) potential host and enabling them to germinate in perfect sync to when a host is present.

Peptide Hormones

Peptide hormones are a growing class of signaling molecules that are short chains of amino acids. In contrast to classical plant hormones that are products of secondary

metabolism, peptide hormones are encoded by genes and synthesized via the secretory pathway. Systemin, the first plant peptide hormone to be identified, is an 18 amino acid peptide that is produced when a tomato leaf is wounded and is involved in mediating systemic mortality signaling. Since that time, different families of peptides were identified as Clavata3/Embryo Surrounding Region-Related (Cle), Phytosulfokine (Psk), Rapid Alkalinization Factor (Ralf), And C-Terminally Encoded Peptide (Cep). Unlike animal peptide hormones, which can arise from many different precursor proteins, plant peptide hormones are often derived from larger preproproteins of which the functional peptide is produced by proteolytic processing and various post-translational modifications such as tyrosine sulfation, proline hydroxylation and arabinosylation. These modifications are often critical for biological function. By and large, peptide signaling is mediated by cell-surface receptor kinases most notably by leucine-rich repeat receptor-like kinases (LRR-RLKs). Ligand binding activates the cytoplasmic kinase domain of the receptor, which in turn starts phosphorylation cascades that ultimately regulate gene expression or cellular processes. Members of the Clavata Peptide Family Are Responsible For Controlling Maintenance Of Meristems, Where Clv3 Limits The Number Of Stem Cells In Shoot Apical Meristems Via Interaction With The Clv1/Clv2 Receptor Complex. This Peptide Family Shows Functional Diversification As Related Cle Peptides Regulate Root Meristem Activity, Vascular Development And Nodulation In Legumes. Peptide Hormones Epidermal Patterning Factors (Epfs) are involved in the coordination of stomatal development through the regulation of asymmetric cell divisions in the epidermis. They work with ERECTA family receptor kinases to maintain appropriate stomatal density and distribution to balance gas exchange and water loss.

Mechanism of Action of Growth Regulators

Explanation about how growth regulators work

Following the Signalling Process

The initial step in plant hormone action is perception of the signal by specific receptor proteins. The cellular localization and signaling mechanisms of different hormone receptors mirror the diverse nature of plant hormones. A family of endoplasmic reticulum-localized receptors, such as ETR1 (ETHYLENE RESPONSE1) and its

homologs perceive ethylene. These copper-binding proteins act as negative regulators of ethylene signaling, in which ethylene binding to the receptor causes, the inhibition to be released and downstream signaling transduction can proceed. Such "relief of inhibition" mechanisms are atypical of hormone signaling. Auxin receptors overlap by the groups of nuclear TIR1/AFB F-box proteins and plasma membrane-localized ABP1. TIR1 is an auxiliary factor of an SCF ubiquitin ligase complex that promotes degradation of Aux/IAA repressor proteins when bound by auxin. ABP1 pathway contributes to rapid, non-transcriptional regulatory effects on ion transport and cell expansion, and this nuclear path is tightly regulated of transcriptional responses. GID1 (GIBBERELLIN INSENSITIVE DWARF1) is part of a family of soluble GA receptors that change conformation when induced by GA. This enables interaction with DELLA repressor proteins, which in turn are targeted for ubiquitin-mediated degradation and relieve transcriptional inhibition on GA-repressing genes. Brassinosteroids are recognized by BRI1 (BRASSINOSTEROID INSENSITIVE1), a plasma membrane leucine-rich repeat receptor-like kinase. Binding of BRs leads to the interaction of BRI1 with the co-receptor BAK1, the activation of intracellular kinase domains and the start of phosphorylation cascades that eventually lead to regulation of nuclear transcription factors such as BES1 and BZR1. Among abscisic acid (ABA) receptors are the PYR/PYL/RCAR protein family members that bind ABA and interact with and inhibit the activity of PP2C phosphatases. This releases inhibition of SnRK2 kinases that phosporylate downstream targets, including transcription factors and ion channels, mediating both gene expression and stomatal closure responses to ABA. Cytokinin sensing uses a two-component signaling system adapted from bacterial signal transduction. The hybrid histidine kinase receptors (AHKs) undergo autophosphorylation after binding to cytokinin, which passes the phosphoryl group to histidine phosphotransfer proteins (AHPs) and subsequently to response regulators (ARRs) that control gene transcription.

Signal Transduction Pathways

Plant hormones trigger complex signal transduction cascades to amplify and specify the initial signal after receptor activation. In addition, there are multiple components such as second messengers, protein kinases/phosphatases, and transcriptional

regulators mediating these pathways. Calcium ions, cyclic nucleotides, phosphoinositides, and reactive oxygen species257389 are some examples of second messengers which are involved in plant hormone signaling. Calcium acts as a general second messenger in a series of hormone pathways, with certain signatures of amplitude, duration, and localization encoding specific stimuli. Signature information is interpreted by specialized calcium-binding proteins such as calmodulin and transmitted to downstream components. Protein phosphorylation cascades are central to hormone signal transduction. Further, mitogen-activated protein kinase (MAPK) modules composed of sequentially activated kinases (MAPKKK '! MAPKK '! MAPK) function in multiple hormone pathways, including ethylene, auxin and JA signaling. Scaffold proteins, subcellular compartmentalization and feedback regulation maintain the specificity of each cascade. For hormones such as auxin, GA, JA, and brassinosteroids, the ubiquitin-proteasome system is employed to degrade signaling repressors. In example auxin, interaction between TIR1/AFB receptors and Aux/IAA repressors is induced by hormone and leads to polyubiquitination and degradation of Aux/IAA. It releases AUXIN RESPONSE FACTOR (ARF) transcription factors from inhibition, thus permitting auxin-responsive gene expression. Hormone crosstalk is mediated at several levels in signal transduction networks. Indeed, components are shared between pathways, as is the case for JAZ repressors targeted by both JA and GA signalling (Pérez et al., 2017; De Brum et al., 2021). Alternatively, pathways can converge on shared targets; many transcription factors are regulated by several hormones. Such interconnectivity allows for crosstalk to integrate different signals to adjust plant responses. Besides, most hormone signaling pathways are self-limiting feedback regulated in order to maintain homeostasis. For example, the hormone auxin triggers expression of genes in the Aux/IAA class whose protein products antagonize auxin signaling. At the same time, cytokinin stimulates the expression of type-AARR genes that encode negative regulators of the cytokinin response. Such negative feedback loops help to inhibit overactivation of the pathway while allowing adaptation of response.

Transcriptional Regulation

Most hormone signalling pathways have transcription factors as their end targets that can alter transcription of genes. Each hormone activates a particular battery of

transcriptional regulators but there is considerable overlap between the pathways. Auxin Signaling by ARF6 and ARF7ARFs act by binding to AuxREs of target genes, thereby facilitating auxin-regulated transcription. In Arabidopsis, 23 ARFs exist as activators or repressors. Assuming low auxin levels, ARFs associate with Aux/IAA repressors to repress transcriptional activity. Auxin-mediated degradation of the Aux/IAA repressor leads to the release of ARFs to regulate target gene expression. Gibberellin-induced responses are regulated by GAMYB transcription factors and DELLA proteins. DELLAs are repressor proteins and degrade in GA perception-dependent manner to relieve inhibition of GA-responsive genes. For example, in cereal aleurone cells, GA-activated GAMYB interacts with GA-response elements (GARE) in á-amylase gene promoters, indicating direct transcriptional regulation (149). Cytokinin action is mediated through a phosphorelay that eventually leads to the activation of type-B ARR transcription factors that bind to cytokinin response elements and regulate gene expression. The primary genes responding to cytokinin are type-A ARRs (which establish negative feedback) and cytokinin response factors (CRFs) that mediate secondary transcriptional responses. A wide variety of transcription factor families are activated by the core ABA signaling module, including ABA-responsive element binding factors (ABFs/AREBs). These bZIPs bind to ABA-responsive elements (ABREs) in the target promoters, which lead to the mediation of transcriptional co-regulators and chromatin modifiers, allowing the activation of ABA-responsive genes. Ethylene signaling converges on the EIN3/EIN3-LIKE (EIL) transcription factor family. EIN3 is subjected to degradation through EIN3-BINDING F-BOX PROTEINS (EBFs) in the absence of ethylene. Upon binding ethylene, it stabilizes EIN3, which transcribes ETHYLENE RESPONSE FACTOR (ERF) genes involved in secondary-response gene regulation. The perception of JA leads to the release of bHLH transcription factors, such as MYC2, from binding to JAZ repressors to activate jasmonate signaling. MYC2 indeed acts as a regulatory hub, positively modulating wound response genes, but negatively modulating pathogen defense genes to strategically rewire JA-dependent transcription. Brassinosteroid-activated transcription factors BES1 and BZR1 associate with E-box and BRRE elements, respectively, in target promoters to recruit histone modification enzymes that modify chromatin structure.

Such epigenetic regulation mediates activation and repression of BR-responsive genes, illustrating t mplexity of hormone-mediated transcriptional regulation.

Regulation of Transcription and Post-Transcriptional Events

Although transcriptional regulation is the main mode through which hormones act, plant growth regulators are also capable of affecting post-transcriptional and translational regulation of gene expression (additional layers of regulation). Auxin regulates mRNA stability via RNA-binding protein-mediated interactions. For example, auxin promotes the degradation of SMALL AUXIN UP RNA (SAUR) transcripts encoding short-lived proteins responsible for mediating cell expansion. This mechanism allows quick modulation of auxin responses without transcriptional changes. Various hormones regulate alternative splicing of pre-mRNAs to produce transcript isoforms with distinct functions or stability. ABA controls splicing factors to regulate the processing of stress-induced transcripts. Likewise, temperature-dependent alternative splicing of clock gene transcripts links circadian rhythms and environmental conditions. MicroRNAs (miRNAs) are emerged to be significant post-transcriptional regulators of multiple hormone pathways. Auxin stimulates miR393 expression, whose targets include TIR1/AFB receptor transcripts, allowing for a negative feedback loop. In contrast, miR160 and miR167 repress specific ARF transcripts, introducing an additional layer of complexity in auxin response regulation. Similar miRNA-mediated regulation takes place in ABA, cytokinin and ethylene signaling networks. Another layer of regulation is translational control, which is especially apparent during stress responses. ABA triggers GCN2 kinase, leading to eIF2á phosphorylation which reduces global protein synthesis but promotes translation of stress-responsive transcripts. Another mechanism is energy conservation at the expense of adaptive responses. The effect of hormones on the stability of specific proteins extends beyond the classical degradation of signaling repressors. PTMs such as phosphorylation, ubiquitination and SUMOylation influence protein turnover, function, and interaction. For instance, the bin2 kinase functions to phosphorylate the BES1/BZR1 transcription factors, tagging them for destruction and blocking BR signaling.

Transport and Spatial Regulation

Hormone signaling has to be combined with the spatial distribution of hormones in plant tissues to fully understand the nature of hormone action. Localized biosynthesis, degradation, and transport also generate hormone gradients that direct development

and synchronize responses among tissues. For instance, auxin transport is an example of directional hormone movement. These theories were strengthened when the chemiosmotic idea revealed that auxin - in cheif the protonated form (IAAH) - could diffuse into cells from the acidic apoplast. Within cells (pH <"7.0), auxin dissociates into the anionic form (IAA-), and therefore requires carrier proteins for its efflux. The polar localization of PIN-FORMED (PIN) efflux carriers on certain faces of the cells generates directional auxin transport, which gives rise to gradients crucial for developmental patterning. In addition to the PINs, the auxin transport machinery also comprises high-capacity auxin efflux ABCB transporters and AUX1/LAX influx carriers that mediate auxin uptake. Tissue-specific differential expression and localization of these transporters create patterns of auxin distribution that regulate processes ranging from embryogenesis to organogenesis. Hall and collaborators point out that cytokinins are mainly transported on their ribosides through the xylem (from root to shoot), with recent evidence indicating that ABCG14 transporters mediate their root-to-shoot translocation. In contrast to this nicely regulated auxin transport system, cytokinin translocation seems less directionally regulated, while local biosynthesis and degradation create functional gradients. Gibberellins are transported in both xylem and phloem in both active and inactive forms. Recently, NPF and SWEET transporters that can translocate gibberellins across membranes have been characterized, offering insights into GA transport processes. Transported biologically inactive GA precursors become activated locally, enabling spatial control over GA responses. Even recently identified hormones, like strigolactones, have specialized transport systems; for example, strigolactone transport from roots to shoots is mediated by the PLEIOTROPIC DRUG RESISTANCE1 (PDR1) ABC transporter. This root-to-shoot communication of upward movement portrays environmental status at root to shoots and is able to coordinate branching and resource availability. ABA transport by both passive diffusion of the protonated form (left) and active transport by ABCG and NPF transporters (right). Under drought conditions, ABA is synthesized in vascular tissue, from where it is distributed to guard cells to mediate stomatal closure. Xylem long-distance ABA transport signals root hydration status to shoots, enabling integrated plant–water relations.

Applications of Growth Regulators in Agriculture and Horticulture

Crop Yield Enhancement

Manipulation of physiological processes controlling yield components with plant growth regulators offers multiple approaches for increasing agricultural productivity. These applications are now an indispensable part of modern crop management systems. Gibberellins are widely used to enhance berry size in seedless grapes. With applications of GA3 at determining developmental stages, fruits are encouraged to divide and stimulate cell expansion, allowing a developing cluster to become larger and more marketable. Applications for Citrus: Comparable applications have been performed with citrus fruit, where they are used to avoid rind aging and senescence and eventually increase the marketable period. Synthetic cytokinins such as 6-benzylaminopurine (6-BA) can also regulate cell division, and these compounds applied during fruit development also enhance fruit size and quality in apple, pear, and stone fruits. In general, cytokinin applications can break apical dominance, resulting in the development of lateral buds which can lead to more productive branches in a number of crops. Ethephon (ethylene-releasing compound) can decrease rice stem elongation and strengthen culms when it is applied at a suitable growth stage in cereal crops. This minimizes lodging (falling over) in wheat, barley and rice — especially under high-nitrogen conditions that usually promote excessive vegetative growth. Preventing lodging results in a larger number of plants developing in an upright position, which allows for mechanical harvest and reduces yield losses.

MCQs:

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?**

a) 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

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

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.
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 CULTURE

Objective:

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

Unit 04: Callus Culture

Definition and Overview of Callus Culture

Callus culture is one of the basest techniques for plant tissue culture, the foundation of many plant biotechnology applications. Callus is essentially found as undifferentiated mass of dedifferentiated plant cells that forms in response to wound or hormonal stimuli under controlled in vitro conditions. This growing tissue is made of parenchymatous cells without the specialized functions and arrangement of intact plant organs. Callus formation, or callogenesis, is essentially when specialized cells lose their distinct identity, re-enter the cycle of cell division, and proliferate into a lump of murky cells. The idea of callus culture was established in the early 20th century when pioneers such as Gottlieb Haberlandt envisioned the culture of isolated plant cells in artificially prepared media. However, the standardization and widespread adaptation of callus culture techniques didn't occur until the 1950s and 1960s, when both plant growth regulators were discovered and appropriate nutrient media formulations developed. The experimental framework provided by scientists such as Folke Skoog, Carlos Miller and Toshio Murashige lifted callus culture from a theoretical idea to a practical laboratory technique. Callus cultures are usually initiated with the selection of suitable explants pieces of plant tissue - obtained from different organs like leaves, stems, roots, or reproductive tissues. According to plant tissue culture study explants are surface sterilized to kill any microbial contaminants and are then inoculated onto nutrient media with different combinations of plant growth regulators, the most common being auxins and cytokinins. Thus, the ratio of these hormones is critical for callus formation over organogenesis or embryogenesis. Proliferation in callus becomes

noticeable under ideal conditions with several days to weeks leading to the development of small translucent protrusions that eventually emerge as larger colonies. The totipotency of plant cells is one of the most critical features of callus culture; despite the differentiation state, they can re-differentiate into any cell type and produce whole plants. This unique feature is what sets plant cells apart from most animal cells and is the biological basis for plant regeneration via tissue culture. Due to the fact, functionally callus cells are totipotent, they are very good experimental systems to study fundamental biology of plant development, somaclonal variation, secondary metabolite biosynthesis, and also for genetic transformation. Callus cultures vary substantially in morphology, physiology, and biochemistry, being influenced by factors including plant species, initial explant, medium composition, and environmental conditions. Types of callus that retain their proliferative capacity indefinitely can be continuously subcultured (so-called continuous callus cultures). These long-term cultures are a precious resource for ongoing experimental and commercial purposes. In contrast, other callus types stopped differentiating or lost viability after a few subcultures, so that periodic reinitiation from fresh explants was required. The nutritional needs for callus growth usually consists of macronutrients (C, H, O, N, P, K, Ca, Mg, and S), micronutrients (Fe, Mn, Zn, B, Cu, and Mo), vitamins (especially B complex), and energy, mainly sucrose. When mixed into a balanced media like Murashige and Skoog (MS), Gamborg's B5, or Nitsch and Nitsch, these components supply the necessary elements for cellular metabolism and growth. The physical state of the medium, liquid or solid with gelling agents (agar, gelrite, etc.) can also affect the aeration, nutrient availability and the overall growth pattern of the callus.

Callus culture has served as a precursor for many biotechnological applications beyond its primary function in plant tissue culture. It is the source of start material for cell suspension cultures, which are vitally important for the large-scale production of useful secondary metabolites. Such data includes genetic material from previously plants that differentiated into different types of cells to form the basis of transgenic plant generation by facilitating easier integration of new genetic material into the callus target tissues. In addition, callus-based systems allow the investigation of somaclonal variation genetic and epigenetic changes that arise during tissue culture that can be harnessed

for crop improvement or studied as model systems of genomic plasticity. Commercial application areas of callus culture are in different fields of agriculture and pharmacology. In agriculture, callus-based micropropagation can offer an accelerated means of generating elite varieties upon which agricultural productivity and global food security depends. Callus cultures of medicinal plants represent a potent source of bioactive compounds, which can help in alleviating the pressure exerted on the natural populations. A cell suspension-based production system is a promising alternative for producing phytopharmaceuticals because compounds in callus culture can be produced under a controlled environment, thereby providing consistent quality, which is a significant challenge in phytopharmaceutical development. Callus culture technology also has some limitations and challenges, despite large-scale application. Somaclonal variation can be advantageous in certain contexts, but potentially undesirable in others, especially with the genetic fidelity goals of conservation work. In addition, some plant species or genotypes are recalcitrant to callus induction or regeneration, requiring special protocols or alternative process solutions. Other technical issues, that require careful management in tissue callus culture systems, include microbial contamination, browning of tissue caused by the oxidation of phenolic compounds, and hyperhydricity (waterlogging of the tissues). Callus culture methods are evolving along with research in plant biotechnology; adopting new techniques and technologies as they develop. Callus cultures have been made more efficient and reproducible by the incorporation of bioreactor systems, automated handling, and non-invasive monitoring. Likewise, the use of molecular biology tools and -omics approaches (genomics, transcriptomics, proteomics, and metabolomics) has facilitated the detailing of the cell and molecular events underpinning callus development and differentiation, making for increasingly targeted and robust applications of this general technique.

Types of Callus

The traits of callus tissues vary with the factors involved in their formation and development. Callus types have been classified from different aspects such as morphology, growing model, cellular constituents, regeneration ability, and functional characteristics. Understanding these differences is essential for research applications as well as commercial exploitation of callus cultures. From the morphological perspective, the callus can be categorized to broadly compact (hard) and friable

(soft). There are tightly held up cells with very few spaces in between intercellular spaces. This type atypically grows slower but is preserved morphology wise after subculturing. In contrast, friable callus is characterized by loosely arranged cells with large intercellular spaces, resulting in a soft, crumbly texture that easily breaks apart when handled. Friable callus has faster growth rates, and is therefore preferred for the establishment of cell suspension cultures because it can be more easily dispersed when introduced into liquid media. Callus morphology is widely influenced by the concentration of growth regulators, specifically the ratio of auxin to cytokinin, and high concentrations of auxins are generally required for friability. Embryogenic vs non-embryogenic callus is another main classification Embryogenic callus has the unique ability to develop into somatic embryos, embryo-like structures derived from somatic cells instead of zygotes. These calli are frequently observed on predisposed explants as nodular and compact masses, which exhibit a characteristic yellowish-white garb and are made of small cytosaineous cells agglutinated in embryogenic cell aggregates. With suitable conditions, embryogenic callus can undergo a series of developmental stages resembling zygotic embryogenesis, completing with the formation of whole plantlets (4). Nonembryogenic callus, on the other hand, does not show this direct embryogenic capacity and is composed mainly of larger, highly vacuolated cells that are randomly organized. The embryogenic competence depends on the source of the explant (with reproductive tissues frequently producing more embryogenic callus), the genotype, and the culture conditions. Depending on the origin and cellular content, callus is categorized as homogeneous or heterogeneous. The homogeneous callus is formed from cells of same type and developmental stage and is derived from tissues having uniform population of cells like leaf mesophyll or stem pith. Whereas organogenic callus displays uniform morphological and biochemical characteristics throughout its mass, heterogeneous callus contains a mix of cell types in different stages of differentiation, mirroring the heterogeneity of the source explant or the presence of differentiation events that occurred spontaneously in culture. Indeed, once a single mass of callus has formed, different growth rates, colors, and textures at various points may reflect such heterogeneity. Callus pigmentation offers an additional classification criterion whereby calli have been classified by their pigmentation as chlorophyllous (green), anthocyanin-rich (red or purple), carotenoid-containing (yellow

or orange) and non-pigmented (white or cream). Chlorophyllous callus, which can photosynthesize due to the presence of chloroplasts, is derived from leaf explants or produced after exposure to light. The anthocyanin-rich callus accumulates these flavonoid pigments in response to stress factors or as part of their normal secondary metabolism. Likewise, the biosynthesis of these yellow-to-orange terpenoid compounds is represented in carotenoid-containing callus. Non-pigmented callus shows little accumulation of pigment and is typically found in dark-maintained cultures or in cultures from non-photosynthetic tissue.

Based on their growth dynamics, callus cultures can be divided into primary, established and habituated cultures. The term primary callus is used to refer to the first proliferative tissue, which develops directly from explants and retains some of the properties of its tissue of origin. This callus was previously established from tissues, has subcultured several times, and adapted to growth in vitro, and often has a more homogenous morphology. After having the ability to thrive in the absence of exogenously supplied plant growth regulators, habituated callus has obtained an autonomous capacity for hormone biosynthesis or altered sensitivity to endogenous hormones. This acclimatization effect reflects adaptation to long-term culture that can greatly alter the physiological and biochemical characteristics of the callus. On functional basis, callus may be broadly classified into two types according to their secondary metabolites production. For example, productive callus synthesizes and accumulates specialized metabolites of interest, e.g. alkaloids, flavonoids, terpenoids, or phenolics, at a level comparable to or higher than that present in the intact plant. This does not happen with non-productive callus, in which the precursors, enzymes or regulatory factors required for biosynthesis might not be present, or the metabolic pathways are not activated under the in vitro culture conditions. Callus induction of secondary metabolism usually needs specific elicitors, stress conditions or media modifications, which has made it a focus of much scientific research for pharmaceutical and nutraceutical purposes. Callus can be classified as either organogenic or non-organogenic depending on their regenerative potential. When appropriate hormonal signals are in place, organogenic callus can differentiate into particular organs like shoots (caulogenesis) or roots (rhizogenesis). And this property is crucial for plant regeneration procedures in micropropagation and processes aimed at genetic transformation. Organogenesis

refers to the development of organized structures such as shoots or roots from the callus tissue, which is necessary for successful plant propagation, whereas non-organogenic callus does not exhibit this ability and remains in an undifferentiated state even in the presence of external factors that promote organogenesis. Organogenic competence is influenced by the genetic background, explant source, culture history and the applied hormones. There are also specialized types of callus; shooty callus is capable of spontaneous shoot bud formation; rooty callus can form root primordia; and nodular callus is similar in appearance to shooty callus and can be characterized by distinct nodules, possibly indicating regions of increased cellular division or organization. We call this stage 'partially differentiated callus', as it is an intermediate state between fully undifferentiated callus and organized organs, and it provides glimpses of the developmental mechanisms involved in plant morphogenesis. Another dimensionality for callus classification is genetic stability with the categories of stable and unstable (or variable) callus. Mature callus retains genetic identity on prolonged culture terms providing karyotype stability and expression of parent phenotype. This kind is better for applications that need consistency and predictability, like germplasm conservation or controlled production of secondary metabolites. Unstable callus shows somaclonal variation genetic or epigenetic changes that accumulate during the course of culture either in chromosome number, structure or in gene expression. Although problematic for some applications, such variation may be beneficial for crop improvement programs seeking novel traits or increased genetic diversity. Callus material itself can be classified based on its aggregation behaviour in liquid bioreactor systems. In contrast, cells from aggregating callus form stable clusters of cells that do not completely break apart, whereas cells from non-aggregating callus break into single or small groups of cells upon agitation. It has a major impact on growth kinetics and oxygen transfer and, thereby, on the performance of suspension cultures originating from these calli, which in turn determines the favorable establishment of large-scale production systems. An age-based classification separates young from senescent callus. Young callus, early subculture cycles, usually exhibited vigorous growth, high cellular viability and obvious regenerative capacity. After many subcultures, senescent callus typically exhibit lower growth rates, higher cell death, and reduced morphogenic potential. The temporal component of callus formation represents the slowly progressive accumulation of genetic aberrations, epigenetic modifications, and physiological

adaptations with prolonged in vitro conditions, suggesting periodic culture update with fresh explants are required for more protracted research or commercial purposes. The identification and classification of these different types of callus are of practical importance in plant biotechnology. Certain applications need specific callus features - friable callus - for cell suspensions, embryogenic callus - for somatic embryogenesis, pigmented callus - for natural colorants production or organogenic callus - for regeneration systems. Thus, the use of different types of callus, whose unique characteristics and requirements are well understood by the practitioner, expedites all types of plant tissue culture and calls for their extensive adoption in the field of plant fundamental research and biotechnology.

Factors Affecting Callus Growth and Differentiation

A multitude of factors interact and shape the growth and differentiation of callus cultures, determining the developmental leaf area, metabolic profiles and capacity for regeneration. These factors can be classified into genetic, physiological, nutritional, hormonal and environmental factors that play an individual role in callus response. Knowledge of these elements and their interrelationships is important to improve callus culture protocols and to control the callus development in order to achieve the desired objectives. Genetic properties of callus represent the first and primary determinants of their behavior, setting the limits of possible responsiveness within which other factors work. This, however, is not true for all plant species, genera, or even families, as certain taxonomic groups (e.g., numerous Gramineae) are especially recalcitrant. Differences in genotypes can influence callus initiation, growth rates, morphological features, and regenerative potential even among the same species. Indeed, these responses are genotype dependent and likely as a consequence of variations in the levels of endogenous hormones, receptor sensitivities, metabolic pathways, and gene expression profile that together determine the cellular competence for dedifferentiation and the following developmental stages. Comparative genomics and transcriptomics have explored the genetic basis of these differences and the key genes involved in cell cycle regulation, chromatin remodeling, hormone signaling, and stress responses have been identified as determinants of callus formation capacity. The choice of explant is a key initial decision that affects the properties of callus in genetic and physiological ways. Plant organs and tissues are composed of

cells that exhibit various combinations of determination, endogenous hormones, and metabolic states. Meristematic tissues, with their continually dividing and less differentiated cells, tend to form callus more readily than highly specialized tissues. Juvenile tissues exhibit a more pronounced response to callus induction stimuli than mature tissues, which mirrors their higher developmental plasticity. All the same, different tissue types of even the same organ can produce callus with non-identical properties, e.g., leaf mesophyll-derived callus tends to differ from leaf vascular-derived callus in growth rate, friability and potential for differentiation. Both the physiological and developmental state of the explant when excised, for instance, season of collection, the age of the plant, and pre-existing stress conditions, also influence its callogenic response. A primary factor influencing callus growth and differentiation is the nutritional composition of the culture medium, which supplies the building blocks and energy needed for cellular metabolism. Carbon source (usually sucrose or glucose): Provides energy and also serves as an osmotic regulator. The callus growth is greatly influenced by the concentration of carbohydrates, with the best levels for this to occur differing between species, although usually falling between the range of 2-5% (w/v). Hazards of the higher concentrations can incite osmotic stress, resulting in somatic embryogenesis or secondary metabolite production, while lower concentrations can limit growth due to insufficient energy. The nitrogen source reported as mixed N in the form of nitrate, ammonium ionsaffect callus cultures pH dynamics, amino acid synthesis and protein metabolism. Concerning the ratio of these two forms of nitrogen, this is an important factor influencing callus growth and morphogenesis, with higher proportions of ammonium favoring callus proliferation and higher proportions of nitrate favoring differentiation.

Macro and micronutrients are vital for callus development because of their involvement in pathways of metabolism and the activation of enzymes along with their structural function. Phosphorus, potassium, calcium, magnesium and sulfur are involved in energy transfer, osmotic regulation, wall formation and protein synthesis. Micronutrients (including iron, manganese, zinc, boron, copper, and molybdenum) are essential for many enzyme reactions and redox processes, and we need them in sub-millimolar concentrations. Abnormal callus may be formed due to a lack of one or more of these elements or because of an imbalance between element levels. Reduced growth rates

or impaired differentiation capacity may also be induced by the inadequacy of these elements. Proteins, carbohydrates, lipids, and vitamins, especially B complex vitamins (thiamine, pyridoxine, nicotinic acid) which act as cofactors for different enzymes in many essential metabolic pathways, are components normally included in the culture media in order to achieve optimal callus growth (Jiang and Dhi, 1994) that has a significant impact on cell metabolism, while total nutrients needed can differ significantly based on species. Perhaps the most powerful external factors affecting callus induction, proliferation, and differentiation are plant growth regulators (PGRs), chemical messengers that affect gene expression and cellular activities. Auxins, including 2,4-dichlorophenoxyacetic acid (2,4-D), naphthaleneacetic acid (NAA), and indole-3-acetic acid (IAA), usually are responsible for stimulating cell division, initiation of callus, and differentiation of root. Of these, 2,4-DPT is distinctly applicable for callus induction due to its strong dedifferentiation hormone activity and long-term protection against degradation by various enzymes. Cytokinins (kinetin, 6-benzylaminopurine [BAP], zeatin) — cytokinin triggers cell division rates and patterns in coordination with auxins, inducing shoot differentiation. In the context of callus development, a critical factor is the auxin to cytokinin ratio, where high auxin/low cytokinin drives root formation, low auxin/high cytokinin induces shoot formation, and intermediate ratios maintain an undifferentiated callus state. However, while other plant growth regulators are not so commonly used in routine callus culture, the use and effects of other plant growth regulators may be considerable in special cases. Some components of plant hormones are generally responsible for inhibiting callus formation, but promoting cell elongation and also affects some differentiation processes. Check other articles, as abscisic acid generally prevents callus development of somatic tissues but can also stimulate somatic embryogenesis under defined circumstances. Ethylene, generated endogenously by plant tissues in culture vessels, accumulates to levels that can substantially influence callus development, usually resulting in suppressed growth and enhanced senescence. Ethylene inhibitors or adsorbents (e.g., silver nitrate or activated charcoal) can reduce these effects and promote callus proliferation. Brassinosteroids, jasmonates, polyamines, and salicylic acid are other signal molecules that influence callus responses across species, most pertinent to stress tolerance, secondary metabolism or cell-fate decision pathways. The physical condition of the culture medium affects the growth of callus by influencing the availability of nutrients, gas exchange,

and mechanical support. Solid media, usually gellied with agar or gelrite, supply structural support and form sharply defined gradients w.r.t nutrients, oxygen, and metabolic byproducts. Liquid culture provides more consistent nutrient distribution and lower diffusion barriers but necessitates more complex supporting methods (filter paper bridges or rotary agitation). This choice is thus driven by the specific goals of regeneration with solid media more often preferred for well-defined calluses that are regularly chosen as starting material for organ regeneration—and preliminary material for sufficient growth and high homogeneity, a necessary requirement for studies on the biochemistry of the preexisting cell walls or the initiation of cell suspensions. These different gelling agents vary in properties that determine the mechanical firmness of a matrix, the availability of water and the possibility of inhibitory contaminants, leading to a distinct preference for gelling agents with purified options (gelrite) for sensitive types of culture. Light and temperature as main environmental factors are able to modulate callus development significantly, possibly by changing photomorphogenic response, enzyme activities and gene expression. So, by adjusting light conditions such as light intensity, light quality (the spectral composition of the light), and photoperiod, it adjusts chlorophyll synthesis, anthocyanin accumulation, and morphogenetic responses for many callus cultures. Although some callus types benefit from darkness for optimal growth, light stimulation often leads to tissue differentiation, especially shoot formation. Callus cultures generally maintain an optimum temperature of between 22-28 °C, which corresponds to the native habitat temperature of respective plant species, given that deviations from optimum temperature can affect its growth pattern and metabolic activity, inducing heat or cold stress responses. Day and night temperature differences in particular can cause some species to respond developmentally, emulating natural seasonal signals.

The use of volatile compounds, like gaseous atmosphere, may modulate callus metabolism as it becomes either limited in the supply of oxygen required for respiration or excessive accumulation of ethylene or carbon dioxide in a closed environment (culture vessels). The sealing materials we use or our ventilated lids make it possible for gas exchange to occur at the levels needed to maintain proper growth, while a lack of ventilation can make micro-aerobic conditions that bend some specific metabolic pathways. In sealed culture vessels, the relative humidity is often high, and it can also

have an effect on water relations and transpiration rates, which can further influence nutrient uptake and cell expansion in the callus tissues. Electromagnetic fields and sound waves are other physical factors that influence callus formation, although their mechanisms of action and clinical application are still under investigation. In general, the culture medium pH can affect the nutrient availability, enzymatic activities, and cell membrane functions in callus cultures. It is common to adjust the initial pH to a range from 5.6-5.8 prior to autoclaving, which is then altered during the course of culture with different ions being taken up and metabolites released. And because some species/ callus types have specific pH preferences, standard protocols may need to be adjusted. The osmotic potential of the medium, influenced by sugar concentration, salt content, and other solutes, similarly impacts the water relations, cell turgor, and other physiological processes in callus cells. It has been reported in some species that manipulating osmotic conditions (eg, altered sucrose levels or osmotic shock) or adding osmotic agents (eg, mannitol, sorbitol) induces stress responses and increases the production of secondary metabolites or morphogenesis. The characteristics of callus are affected by temporal features such as the period of culture and the number of subcultures due to nutrient depletion, waste product accumulation and cellular aging. Prolonged culture without subculture eventually drains nutrients, dehydrates the medium, and concentrates possibly inhibitory metabolites built up to toxic and/or inhibitory levels, inducing a suite of stress responses that can initiate differentiation or senescence. Subcultured often to a fresh medium, this keeps up with active growth, but over time they might select for faster growing cell line rather than a genetically and biochemically diverse callus. The effectiveness of hormonal treatments is highly dependent on the timing of application relative to the callus growth cycle, as cells in various physiological states respond differently to morphogenic signals. Meanwhile, mechanical factors like pressure from explants preparation, orientation of explants on the medium, and aeration level in fluid cultures act as physical signals that affect callus induction and development. Wounding responses activated by the large-scale disrupting of tissues (during explant preparation) lead to signaling cascades mediated by jasmonates, ethylene and reactive oxygen species to induce cellular dedifferentiation and division. The dimensions and morphology of explants determine the surface area-to-volume

ratio and thus the rate of nutrient absorption and gas exchange; smaller explants are generally favourable for rapid callus initiation but may be more vulnerable to stress-induced injury. Additionally, biotic factors like possible endophytic microorganisms or plant growth-promoting substances that might be leftover from the parental plant could have slight impacts on callus growth. Callus growth can be influenced by endogenous hormone, enzyme, or antimicrobial compound production from internal microbiota that could survive surface sterilization. Similarly, unfinished compounds (e.g., oligosaccharides, glycoproteins, or small peptides), natural organic supplements in plant extracts (e.g., coconut water, casein hydrolysate) can be applied to cell culture systems to modulate the intracellular signaling between cell division and differentiation pathways, through complex mechanisms that cannot be reproduced with a defined media component system. This comprehensive comprehension of these complex influences forms the theoretical basis for the targeted manipulation of callus cultures. This approach allows the systematic tuning of genetic, nutritional, hormonal, and environmental parameters to steer callus formation towards improved biomass yields, effective regeneration, or high accumulation of secondary metabolites. Our findings provide a basis for knowledge-driven approaches to optimizing the culture of callus for use in biotechnological applications that range from crop flourish and conservation, through drug manufacture, to experimental probing of plant developmental processes.

Application of Callus Culture in Plant Regeneration

One of the most important applications of plant tissue culture, therefore is plant regeneration from callus cultures, to produce whole plants from somatic cells through organized developmental pathways. This extraordinary potential, based on the concept of cellular totipotency, has transformed the methods of propagation, conservation, and improvement of different plant species. The regeneration process can take two different developmental pathways: organogenesis, where new organs (shoots and roots) are formed de novo, and somatic embryogenesis, where embryo-like structures with bipolarity are formed, which can germinate directly into whole plantlets. These pathways, though mechanistically distinct, both exploit developmental plasticity of callus cells to reconstruct the complex architectural and functional organization of whole plants. In most cases, organogenesis is a sequential process initiated by sensnickle organ primordia in the callus under the appropriate hormonal level. Shoot organogenesis

(caulogenesis) is typically initiated by passage of the callus to media with higher concentrations of cytokinin, compared to auxin, thus forming a hormonal milieu that accelerates cell divisional patterns favorable to meristem formation. The first response is the formation of meristematic centers (nodules) in the callus mass, and consists of small cells with dense cytoplasm and large nuclei. The initial meristematic foci then arrange into shoot apical meristems, which subsequently differentiate to form both shoot buds and leaf primordia. The nascent shoots grow and form entire aerial organs with leaves and internodes. In contrast, root organogenesis (rhizogenesis) is generally induced on auxin-rich media, after shoot formation in some cases, as a continuation of the wound healing process. In some systems, single- or direct-shoot root regeneration is observed but in most, shoot and root events are temporally and spatially separated, requiring multiple manipulations of the culture to facilitate complete plantlet regeneration. The organogenic pathway is highly variable in plants species and genotypes, and it requires empirical optimization of regeneration protocols. Organogenic potential can be influenced by a multitude of factors including the plant species, the explant source, the type of callus, the culture history, the media composition, and the environmental conditions. In particular cells from juvenile tissues tend to exceed the organogenic competence of mature tissues, and primary callus often outperforms long-established callus cultures that could have accumulated genetic alterations. The duration of the callus phase has a profound impact on the subsequent efficiency of regeneration, and extended periods of undifferentiated growth can attenuate organogenic potential via somaclonal variation or epigenetic changes. This phenomenon, referred to as "culture aging", has practical applications for regeneration-centric applications, and indicates a need to curb the duration of the callus phase if genetic fidelity is of concern. Somatic embryogenesis is an alternative regeneration pathway in which callus cells give rise to embryo-like structures resembling many aspects of zygotic embryogenesis. Such process may take place directly from explant tissues without an intermediate callus phase (direct somatic embryogenesis) or, more interesting and relevant for callus applications, from the well-established cultures of callus (indirect somatic embryogenesis). Previously, the induction of embryogenic callus has generally required specific hormonal treatments (see below) and is characterized by the use of the auxin 2,4-D (often at high concentrations) during the induction but then its removal or diluting and/or decreasing concentrations for embryo

development. During the transformation from undifferentiated callus to embryogenic identity, dramatic alterations in cellular metabolism, gene expression profiles and cytological properties occur, which eventually lead to the formation of embryogenic cell clusters, which separate into the surrounding callus matrix. Once initiated, somatic embryogenesis consists of well-defined developmental stages resembling zygotic embryonic development, such as globular, heart, torpedo and cotyledonary stages in dicotyledonous plants or similar sequential forms in monocotyledonous species. These stages progressively correspond with increased cellular complexity and completion of morphogenetic events, leading to the creation of somatic embryos that have defined apical shoot/root meristems, as well as cotyledons (or scutellum) and an established embryo axis. Most of these embryos can germinate directly into plantlets on suitable media, although they sometimes require a maturation step, hormonal treatments, desiccation, or the addition of abscisic acid to promote uniform development and restore germination behaviour. Somatic embryogenesis has distinct advantages over organogenesis in generation applications such as the generation of whole plantlets in one step (thereby eliminating the requirement for separate rooting treatments), the ability to achieve a high multiplication rate, and the provision of defined shoot-root associations through normal vascular differentiation. Common drawbacks like asynchronous development, abnormal embryo morphology and poor germination can require further optimization steps. The embryogenic pathway is especially useful for species where traditional organogenic methods are inefficient, including many woody plants, cereals, and legumes.

Callus-based plant regeneration has a wide range of practical applications in various areas of plant science and biotechnology. Callus cultures are essential intermediate stages for commercial micropropagation of commercially important plants, especially when direct organogenesis from explants is inefficient or when high multiplication rates are demanded. In general, direct organogenesis without a callus phase is advantageous for commercial uses owing to reduced somaclonal variation, although callus-mediated approaches are usefully applied to species with little or no tendency to regenerate directly. The scalability and cost-effectiveness of callus-based propagation approaches have been substantially improved as the traditional labor-intensive methods have been replaced by bioreactor systems in combination with automated handling

(Wang et al., 2021). Callus-based regeneration systems serve as fundamental platforms for multiple biotechnological applications in crop improvement programs. The introduction of genes through Agrobacterium-mediated transformation, particle bombardment or other means is usually carried out in callus tissue to are regenerated into transformed plants by organogenesis or somatic embryogenesis. Transformed cells are usually selected during the callus stage with selectable markers that provide resistance to antibiotics, herbicides, or other selective agents. Likewise, mutagenesis approaches are usually applied to callus cultures (be it chemical, physical or insertional) to produce genetic variability, which can then be regenerated to develop and screen for the elicited phenotypical changes. Somaclonal variation genetic and epigenetic changes that take place during the tissue culture process is both a challenge and an opportunity in callus-based regeneration systems. Although potentially an obstacle for applications demanding genetic fidelity,15 such variation can be harnessed as a source of new traits for crop improvement. Examples of valuable variants with enhanced disease resistance, abiotic stress tolerance, yield components, or quality traits have been obtained from generations of regeneratorates derived from callus lines in a range of crop species. The work provided insight into genomic plasticity through molecular characterization of the underlying genetic alterations, and produced novel germplasm for breeding programs through systematic screening of callus-derived regenerants. Another important use of callus-mediated regeneration is the production of doubled haploids which is routinely employed in breeding programs of many economically important crops. In fact, the induction of chromosome doubling from callus derived from haploid tissues such as microspores, anthers or ovaries followed by regeneration, can yield fully homozygous plants after one generation. This method greatly shortens generation times by skipping over generations of self-pollination, allowing quickly generation of pure lines that can be used for hybrid or variety development. Doubled haploid lines can be produced at very different rates depending on species and genotypes, but much of this success is critically sensitive to callus culture conditions that not only affect chromosome doubling frequency but also their later successful regeneration. In conservation biology, callus-based regeneration can be an important tool to develop ex situ preservation approaches for endangered plant species, in conjunction with seed banking and field gene banks. For such species, or those with small reproductive output, or special conservation challenges, plants in callus cultures

offer alternative options for germplasm storage. Cryopreservation techniques performed on embryogenic callus or derived somatic embryos allows for long-term storage at ultra-low temperatures and can keep cells viable for decades, all while taking up minimal space and requiring very little maintenance. The genetic stability of long-term cultured callus remains a significant concern that requires constant monitoring and management strategies like periodic regeneration and evaluation of genetic fidelity via molecular markers.

This process of regeneration from protoplast-derived callus is an industrial application as a mediating platform for somatic hybridization and for genetic manipulation. They can be isolated from a range of tissues, fused to generate somatic hybrids or transformed with foreign DNA, with subsequent regeneration of cell walls and the formation of callus under the appropriate culture conditions. The subsequent conversion of calli derived from these protoplasts into whole plantlets ultimately allows for the recovery of genetically modified or hybrid plantlets containing genes from other species or integration for the purpose of novel genotypic constructs. Although technically demanding, protoplast-to-plant systems have been completed in several crop species, leading to novel germplasm development and overcoming of barriers to sexual incompatibility. Beyond these practical implications, callus-based systems for regeneration also offer a simple model for investigating basic plant developmental biology. In a nature reminiscent of primordial chaos to shape, the development of undifferentiated callus into ordered structures reveals the cellular and molecular bases underlying patterning, cellular specification and morphogenesis in plants.; The controlled perturbation of regenerative pathways using chemical, physical or genetic tooling enables dissection of the intricate signaling networks and gene expression cascades that instruct developmental choices. The use of advanced imaging technologies together with molecular markers for certain cell types or developmental stages have allowed us to follow and explore the progressive cellular alterations in regeneration from callus and similar processes furthering our general knowledge of plant development biology. There are numerous challenges with the practical application of regeneration systems based on callus and further research is needed. Genotype-dependent responses pose a major bottleneck, often resulting in only negligible callus generation or regeneration

potential from elite crop cultivars or wild species. This "genotype recalcitrance" requires either the establishment of genotype-specific protocols or genetic factors that improve regenerability in broader germplasm. Developmental asynchrony, which is both a commonality and a complication of somatic embryogenesis, creates issues of scale and standardization, driving research toward synchronization techniques via physical treatment, hormonal pulses or developmental marker. Hyperhydricity (or vitrification), a physiological disorder with water-soaked, translucent tissues with abnormal morphology, remains a pervasive issue in the culture of regenerated shoots from callus cultures, with various approaches involving media composition adjustments, ventilation conditions and/or antioxidant supplementation being used to alleviate it. Regenerated plants quality and performance are major concerns for their practical application. Plants that arise from the callus cultures may be abnormal in many ways, such as displaying altered leaf morphology, reduced fertility, or compromised stress-resistance, which can endanger their agronomic value or ecological impact. They can also result from somaclonal variation, epigenetic alterations, or physiological acclimatization to in vitro conditions. Some of these challenges can be managed to an extent by using acclimation protocols which are characterized by the step-wise exposure of seedlings to the physical and nutrient demands of ex vitro conditions under controlled humidity, light intensities and nutritional regimes, to assist in modulating the switch from heterotrophic to autotrophic growth and development of functional adaptations to natural environments.

Unit 05: Organogenesis and Somatic Embryogenesis

Organs and somatic embryogenesis introductory

Plant tissue culture has developed a new understanding of plant development and has changed agricultural and horticultural practices with the development of micropropagation techniques. Two Fundamental Morphogenic Pathways at The Heart Of These Techniques: Organogenesis And Somatic Embryogenesis These have been described as unique The pathways distinct developmental pathways whereby plant cells can undergo differentiation and organogenesis leading to complete plantlets. Organogenesis is an asexually reproductive phenomenon whereby multicellular structures are developed from cultured cells/tissues without passing

through the embryonic stage (Liu, 2009). Somatic embryogenesis refers to the ability of somatic cells to form embryo-like structures that have developmental similarities to zygotic embryos but do not rely on sexual reproduction. Both processes are astonishing demonstrations of plant development plasticity and totipotency, the ability of a single cell to grow into an entire plant, a characteristic almost exclusive to plants within multicellular organisms. These processes in real life are of great importance to the development of certain biotechnological applications such as clonal propagation, germplasm preservation, genetic transformation, and synthetic seeds. Organogenesis is a process that has been studied since early 20th century when Gottlieb Haberlandt first suggested plant cell totipotency (Haberlandt, 1902). It was not until the 1950s and 1960s, F.C. Steward's pioneering research on carrot somatic embryogenesis, as well as the work of Folke Skoog and Carlos Miller on tobacco organogenesis, that these processes were fully demonstrated and started to be understood in a fundamental way. Their finding that a balance between auxins and cytokinins could determine whether morphogenesis was toward roots or shoots provided the foundation of contemporary plant tissue culture. In the last four and a half years, we've made rapid advances towards understanding the molecular and cellular bases of these coupled processes, uncovering complex regulatory pathways involving gene expression, hormone signaling and epigenetic mechanisms that govern cell fate decisions during plant regeneration. An understanding of this knowledge has facilitated the optimization of culture conditions and the establishment of suitable protocols for an ever-growing number of plant species, many of which were previously recalcitrant to tissue culture techniques. The applications of organogenesis and somatic embryogenesis are not just theoretical; test-tube clones are already being used commercially by fruit growers and biotechnology companies. In agriculture and horticulture, these processes allow the rapid increase of elite genotypes, the production of disease-free planting material, and the preservation of endangered species. In the case of forestry, they permit the wide-scale propagation of superior trees with desirable traits. In transgenic plant breeding, they enable regeneration of plants from transformed cells, aiding genetic augmentation efforts. Moreover, somatic embryogenesis has paved the way for synthetic seed production, which involves encapsulating somatic embryos in a supportive medium that can be handled, stored, and planted similarly to conventional

seeds. These processes also have wider experimental significance: organogenesis and somatic embryogenesis represent great experimental systems for addressing fundamental problems such as cell fate determination, patterning and gene regulation in plant development. Exploitation of these processes in vitro opens new avenues to study the molecular mechanisms of plant morphogenesis, revealing possibilities for basic science and applied biotechnology alike. However, much remains to be done to achieve a successful outcome in organogenesis and somatic embryogenesis. The recalcitrance of many economically important plant species or genotypes to tissue culture greatly hampers the improvement of these species with biotechnological tools. Somaclonal variation, a phenomenon that can occur during the culture process characterized by genetic and epigenetic changes, creates both challenges and opportunities for plant improvement. Additionally, these morphogenic processes often need to be optimized for commercial applications, such as in terms of their efficiency, synchrony, and scalability. Overcoming these obstacles requires a comprehensive understanding of the molecular processes underlying plant regeneration, coupled with the development of novel strategies to modulate these processes. This dynamic restructuring, fueled by the ever-growing merger of genomics, proteomics metabolomics and bioinformatics with traditional tissue culture, continues to expand our horizons as we endeavor to harness and exploit plant morphogenetic processes for scientific and practical needs.

Mechanism of Organogenesis and Somatic Embryogenesis

A brief update on the, mechanistic basis of organogenesis and somatic embryogenesis, two complex cellular and molecular processes that illustrate the unique developmental plasticity of plant cells. At the cellular level, organogenesis usually initiates by the generation of meristematic centers called meristemoids derived from differentiated cells that undergo dedifferentiation and subsequent redifferentiation into organ primordia (Huang et al. 2014). These meristemoids then grow into ordered structures with polarity and histological features typical of shoots or roots. Organogenesis can take place either through a direct pathway whereby the organs are produced ultimately from the explant tissue without an intermediate callus stage or through an indirect pathway in whcih a callus is first formed and subsequenlty, the formation of meristemoids occurs within the callus tissue. Mediated cell-cell

interactions are crucial in plant development, however, the process differs notably from somatic embryogenesis, whereby somatic cells are first reprogrammed to gain embryogenic capacity, then undergo a synchronized expression of an embryonic developmental pathway. This process differs by a series of developmental stages that recapitulate zygotic embryogenesis, showing morphological and molecular characteristics including globular, heart, torpedo, and cotyledonary stages in dicotyledonous plants. Similar to organogenesis, somatic embryogenesis can also be classified as either direct, where embryos develop directly from explant tissue, or indirect, such as when an intermediate callus phase forms. Hormonal control of this morphogenic process has been studied for decades. The classical observation that the auxin:cytokinin ratio determines the developmental fate of cultured tissues; high ratios favor rooting and restrain shoot formation, low ratios promote shoot development and root organogenesis, whereas intermediate ratios elicit callus proliferation, provides a conceptual framework for hormonal control of organogenesis. In somatic embryogeneiss, auxins, such as 2,4-dichlorophenoxyacetic acid (2,4-D), usually plays an important role in the induction of embryogenic competence and the following withdrawal or reduction of auxin normally favours embryo maturation. Yet this rather simplified sketch has recently been considerably detailed and supplemented by studying complex crosstalks mainly between different classes of plant hormones, that is, auxins, cytokinins, gibberellins, abscisic acid, ethylene, and brassinosteroids. Such interactions form dynamic networks of hormones that coordinately regulate distinct cellular processes at different stages of morphogenesis. Auxin, for example, is often required during the induction phase of somatic embryogenesis for cell dedifferentiation and embryogenic competence acquisition, whilst abscisic acid regulates accumulation of storage proteins and desiccation tolerance development, promoting embryo maturation. Genetic and genomic approaches continued to provide crucial insight into the molecular mechanisms that have underknown organogenesis and somatic embryogenesis. Transcription factors are key regulators of pathways associated with these developmental processes. During the organogenesis phase of shoot formation, transcription factors such as the WUSCHEL-RELATED HOMEOBOX (WOX) family members specifying and maintaining meristematic identity and the SHOOT MERISTEMLESS (STM) gene necessary for shoot meristem formation play a prominent role. During shoot organogenesis, cytokinin signaling is

mediated by the type-B ARABIDOPSIS RESPONSE REGULATOR (ARR) family of cytokinin-responsive transcription factors, whereas PLETHORA genes, which are regulated by auxin, are implicated in root organogenesis. Transcription factors associated with zygotic embryogenesis have been shown to act during somatic embryogenesis, with some being essential for somatic embryo development. The LAFL network consists of LEAFY COTYLEDON1 (LEC1) and LEC2, FUSCA3 (FUS3), ABSCISIC ACID INSENSITIVE3 (ABI3), and BABY BOOM (BBM) and governs various aspects of embryo identity and maturation. Known factors, such as BBM, LEC2 and WUS, can ectopically enhance or even induce embryogenic competence in somatic tissues, confirming that they serve central roles in reprogramming cellular identity.

Another key aspect of the molecular control of organogenesis and somatic embryogenesis comes in the form of epigenetic regulation. The process from somatic to embryogenic cell identity and from a differentiated to a meristematic state is associated with marked chromatin remodeling and changes in DNA methylation patterns. Such epigenetic changes can switch on or off critical developmental genes to promote and sustain the cellular fates in question. For example, some repression of embryogenesis-related genes in somatic tissues is mediated by Polycomb Repressive Complex 2 (PRC2), which catalyzes trimethylation of lysine 27 on histone H3 (H3K27me3), a repressive chromatin mark. This repression is often relieved in the acquisition of embryogenic competence, permitting the expression of embryonic genes in somatic contexts. Such changes in DNA methylation have been described at different stages of organogenesis as well as somatic embryogenesis and global DNA demethylation are typically coupled to dedifferentiation and redifferentiation at the cellular level. Together, the genetic and epigenetic factors establish a network of regulation that underlies the extensive developmental plasticity of plant cells, allowing them to achieve their totipotent nature in the presence of relevant signals and environment. Cellular and tissue environment is one of the most significant factors determining the fate for organogenic and somatic embryogenic pathways. Chemical signalling between cells by mobile signals such as peptides, small RNAs, transcription factors and hormones, orchestrates cellular behaviours in developing tissues. Morphogenic processes are also influenced by physical factors, including cell wall

properties, mechanical forces, and tissue polarity. The extracellular matrix consists of cell wall components and soluble secreted molecules that can provide structural scaffolds and regulatory signals that influence cell division patterns, differentiation, and morphogenesis. In culture systems, the medium, including its composition (nutrients, vitamins, amino acids, and growth regulators), creates an artificial environment that can have a profound impact on development. The developmental age, nutritional status, and stress condition of the explant will also impact on the responsiveness of the explant to morphogenic signals (Guan et al., 2020). This knowledge was instrumental in building up effective protocols based on organogenesis and somatic embryogenesis. In recent times, detailed analyses of transcriptomic, proteomic, and metabolomic changes during the different stages of organogenesis and somatic embryogenesis, thus far not possible, have become available due to advances in high-throughput molecular techniques. These investigations, facilitated by advancements in omics technologies, have uncovered dynamic remodeling of gene expression, protein levels, and metabolite profiles, allowing for the characterization of molecular signatures underlying developmental transitions. For instance, transcriptomic analyses have characterized classes of genes expressed uniquely at the induction, development, and maturation stages of somatic embryogenesis, such as those involved in stress responses, hormonic signaling, cell cycle control, and developmental programming. Proteomic studies have added to these findings by uncovering changes in protein abundance and post-translational modifications (PTMs) that may not be evident at the transcript level. Metabolomic analyses have also highlighted shifts in primary and secondary metabolism based on developmental stage, such as alterations in carbohydrate metabolism, amino acid biosynthesis, and accumulation of storage compounds during embryo maturation. The development of high-throughput sequencing methods, the combination of these multi-omics data with spatial and temporal resolution are offering unprecedented insights into the molecular networks governing plant morphogenesis thereby allowing more efficient targeted approaches to improve both the efficiency and applicability of plant regeneration systems.

Techniques and Applications

An important step in the in vitro methods of organogenesis and somatic embryogenesis is the selection and preparation of explants. Regenerating potential is greatly affected by explant choice, genotype, developmental stage, physiological condition and

genotype all affect molding responses. Explant sources include segments of leaves, stem internodes, root sections, cotyledons, hypocotyls, anthers, microspores, immature embryos and flowering tissues. Explant surface sterilization is an important step to ensure that microbial contamination will not affect explant culture in vitro. After sterilization, the explants are generally cultured on solid or liquid medium supplemented appropriately with macronutrients, micronutrients, vitamins, carbon source (sucrose as carbon source used commonly), growth regulators and solidifying agents (for solid medium). The composition of the culture medium is adapted according to the plant species, the genotype, and the morphogenic response desired, with the Murashige and Skoog (MS) formulation being widely used and frequently customized. Light conditions, temperature, and relative humidity are also strictly controlled in the physical culture environment to create optimal morphogenic responses. Direct organogenesis is a process where explants are cultured in media supplemented with specific concentrations and types of plant growth regulators, especially auxins and cytokinins, to develop shoot or root primordia directly from explant tissue. Shoot organogenesis induction typically needs relatively high cytokinin: auxin ratios, common cytokinins used being 6-benzylaminopurine (BAP), kinetin, zeatin and thidiazuron (TDZ). After shoots and leaves have developed, they can be moved to rooting media with auxins (such as indole-3-butyric acid [IBA] or naphthaleneacetic acid [NAA]) used to stimulate root growth and plantlet formation. Indirect organogenesis includes an intermediate callus phase in which explants are cultured first on media that induce callus formation (typically containing balanced concentrations of auxins and cytokinins). Afterwards, the callus is placed in media with the suitable hormonal compositions to develop shoots or roots. The first step involves the proliferation of undifferentiated callus tissue that can be used as a source of multiple regenerants, but it is more susceptible towards somaclonal variation than direct organogenesis. Somatic embryogenesis techniques are analogous to organogenesis techniques with respect to explant preparation and general culture conditions, but with specific adjustments to induce embryogenic rather than organogenic development. In somatic embryogenesis, induction is generally achieved by exposing explants to stressful conditions (typically, a high concentration of auxins (particularly, 2,4-D), osmotic stress (resulting from a high concentration of sucrose or osmotically active compounds, e.g., mannitol), heavy metal stress, and temperature

stress. This phase upon these environmental conditions induces a cellular reprogramming, granting the embryogenic competence. Embryogenic cultures can be maintained by repeated subculture on auxin-containing media after induction, or they can be placed on development and maturation media where auxin is decreased or removed and the addition of other plant growth regulators, including abscisic acid, can be made to promote embryo maturation. While synchronization of embryo development is difficult in many systems, techniques such as size fractionation, density gradient centrifugation or implementation of temporary immersion systems that will allow periodic contact with liquid medium can help negate this issue. Mature somatic embryos can be germinated directly on suitable media, or subjected to desiccation treatments that simulate natural seed maturation processes and often improve germination rates (Dixon and Gonzalo, 2020).

Micropropagation is one of the most successful commercial applications of organogenesis and somatic embryogenesis, allowing for rapid, large-scale propagation of selected plant genotypes available. Typically, the process involves several steps: initiation, propagation,(shoot or somatic embryo), rooting or somatic embryo germination and acclimatization of in vitro plantlets to ex vitro conditions. Micropropagation has multiple benefits compared to traditional propagation methods, such as increased multiplication, the ability to produce plants year-round without being limited by seasonal constraints, lower space requirements, and disease-free plant production, as well as the possibility of propagation of difficult-to-multiply species that cannot be reproduced. “Micropropagation has wide-ranging applications in horticulture, including ornamentals, fruit, timber, and labor-intensive monoculture crops such as banana, potato, and sugarcane. Somatic embryogenesis is mostly preferred for entire large-scale applications, as it bypasses the early organ-specific stages of regeneration and can be scaled up to volumes of greater than 1 liter and single-cell origin nature of somatic embryos gives them a seam of developmental similarity to seeds. There are a number of well-established regeneration protocols for transformed plants, based largely on organogenesis and somatic embryogenesis. In order to recover transgenic plants, the transgene of interest must be integrated into the genome of the plant cell, and the transformed cells must be able to regenerate via organogenesis or somatic embryogenesis after the introduction of foreign DNA into plant cells by

Agrobacterium-mediated transformation, biolistics, or protoplast transfection. Selectable marker genes, for example antibiotic or herbicide resistance genes, provide for the ability to select transformed cells on regeneration media containing the selective agents. Recalcitrance to tissue culture often acts as a bottleneck in the genetic engineering of many key crops, with very diverse transformation-regeneration systems reported in several species and genotypes. Research is also underway aiming to not only achieve regeneration using key developmental regulators but also optimizing the culture conditions and genotyping to identify those with high regenerative capacity. Recent developments in genome editing technologies (especially those based on CRISPR-Cas systems) have only increased the need for efficient regeneration systems that would allow recovery of genetically modified plants without the integration of foreign DNA, highlighting renewed efforts to develop and optimize plant regeneration protocols across diverse species and genotypes. The generation of synthetic seeds is a typical utilization of somatic embryogenesis which distinctively merges the developmental capabilities of somatic epitomes with the conveniences of seed-based multiplication. Synthetic or artificial seeds are usually produced by coating somatic embryos with an inner matrix of sodium alginate polymerized with calcium chloride to make a hydrogel capsule. Nutrients, growth regulators, fungicides, etc. can also be included within these capsules to improve embryo survival,.

Unit 06: Micropropagation

Introduction to Axillary Bud Culture

14- Final Advanced topic: Micropropagation / MultiplicationMicropropagation is one of the most important application of plant tissue culture technology,providing a new revolutionary method for plant multipication that is highly effecient and large scale than conventional propagation methods. There are different types of micropropagation methods, but the axillary bud culture method is the most widely used and commercially feasible method due to its reliability and genetic stability. This technique manipulates the growth potential of the existing meristematic tissues (axillary buds which would otherwise remain quiescent in the leaf axils of plants) by removing them from the influence of apical dominance and inducing their growth into whole plantlets in in vitro conditions. Because the active growth point(s) give

rise to new shoots, the process begins with the accurate placement of dormant axillary buds below an active shoot; however, the technique requires careful management of plant hormones to induce new shoots, which overcome apical dominance, a process that the main growing shoot of a plant often does to suppress the growth of lateral buds. In intact plants, auxins produced in the apical meristem account for this dominance by preventing the growth of axillary buds. Specific cytokinins can enhance shoot formation in nodal segments. When nodal segments containing inactive axillary buds are isolated and cultured on media supplemented with suitable cytokinins, the inhibition caused by apical dominance is lifted and the axillary buds develop into shoots. The new nodes can then be repeatedly subcultured to create a cascade of multiplication that produces thousands or even millions of genetically identical plants from a single explant. The historical axillary bud culture began with the studies of Morel in the 1960s, who was the first to using this technique as a means of virus elimination in orchids. The method was then further developed and adapted to many plant species and became a multi-staged advanced protocol with 4 steps (aseptic cultures establishment, shoots multiplication, roots induction and plantlets acclimatization to ex vitro milieu). Carefully optimized culture conditions, from media composition, plant growth regulators, environmental factors (light and temperature), and handling procedures to ensure maximum rates of multiplication and genetic/growth stability, are required for each of these stages. Compare with adventitious regeneration or somatic embryogenesis, what sets axillary bud culture apart from other micropropagation systems is the way in which pre-existing meristems were already in place and utilized rather than inducing de novo organogenesis or embryogenesis. This trait provides considerable ease and genetic stability because it reduces the chance of somaclonal variation, genetic or epigenetic modifications that can take place in tissue culture and lead to the production of off-type plants. Axillary bud culture retains a very high level of genetic fidelity, making it exceptionally useful in commercial practice when uniformity is desired, most notably ornamental horticulture, forestry, fruit crops, and conservation of some rare or endangered species. Physiologically, axillary bud culture can be understood as a delicate balance of plant hormones, specifically auxiliary hormones like auxins and cytokines, controlling bud dormancy and emergence. Cytokinins are primarily responsible for breaking bud dormancy and stimulating bud growth into shoots and auxins are normally antagonistic to this action, but must be

present for roots to form later. The various phases of the procedure of micropropagation are only successful when a specific combination and concentration of these hormones was achieved at the right time. Studies have recently provided a better understanding of the molecular basis of the dormancy and activation of axillary buds, and recent studies found novel genes and signaling pathways that regulate axillary buds, which may serve as references for further improvement of the axillary bud culture. Axillary bud culture has limitations despite being widely adopted, and there is still room for research and innovations in this area. Such limitations are species specific, but include recalcitrance to in vitro culture; hyperhydricity (also known as vitrification), such that tissue becomes waterlogged and translucent; microbial contamination; and difficulties in acclimatizing plantlets to ex vitro environments. A common group of assumptions needs to be able to make optimizing procedures effectively feasible, such as the need for some form of biological base, thus accounting to refine biological strain improvement strategies with empirical data. Moreover, high multiplication rates need to be achieved, and labor costs minimized to make axillary bud culture systems economically viable, therefore their automation and the development of more efficient culture systems, such as the temporary immersion bioreactors, have been pursued. Today, with the concern towards sustainable agriculture, conservation, high demand and cultivation of specific plant products, the feasibility of axillary bud culture have become even more significant. In vitro propagation allows for expeditious multiplication of elite genotypes possessing desirable traits, lowers the risk of long-distance quarantine when exchanging plant material internationally, and in vitro gene banks allow for conservation of rare germplasm. In addition to that, the potential of many advanced biotechnological applications such as genetic transformation, polyploid induction, and somatic hybridization rely on it, hence contributing positively to crop improvement programs worldwide.

Explant sources for the culture of axillary buds

The choice of suitable explant material is one of the most important factors affecting axillary bud culture, controlling not only the establishment rate of aseptic cultures, but also their potential for further proliferation and regeneration. Nodal segments containing dormant axillary buds are in general used as explants for axillary bud culture, however shoot tips that consist of the apical meristem can be used depending on the specific

goals of the propagation program. The most favorable explant sources should possess high regenerative potential, low risk of contamination, year-around availability and uniform response to in vitro environments; however, such characteristics differ among certain plant species and propagation environments, guiding the selection process. Previously, the most common explant source for bud culture initiation has been between active shoots of field-grown plants. Additionally they have the benefit of strong growth potential and physiological competence to expand quickly upon exposure to relevant culture conditions. But they are also associated with numerous problems with respect to contamination control since field-grown plants contain a broad spectrum of microorganisms on their surfaces and in their tissues. Such explants must also be harvested in accordance with seasonal availability, further curtailing their potential for running year-round propagation programs, especially in temperate areas where periods of active growth may be confined to certain months of the year. Another source of explants that may alleviate certain of these limitations might be greenhouse-maintained mother plants. It is possible to significantly minimize the contamination load by growing stock plants in a controlled environment, where their exposure to pests and pathogens is reduced. Moreover, greenhouse cultivation provides the ability to manipulate the growth environment to maintain vegetative growth throughout the year, making explant material available all year round. Most commercial micropropagation labs have stand-alone greenhouses for this purpose, with rigid sanitation procedures in place and preventative treatments to minimize contaminant introduction. Plants can additionally be pretreated with systemic fungicides and bactericides for several weeks prior to explant collection to minimize endogenous contamination, but these treatments must be carefully chosen so as not to negatively affect subsequent response in vitro. However, woody perennial species distinguish themselves with their typical pronounced growth trends and, alongside high concentrations of phenolics that negatively affect in vitro growth, require dedicated approaches towards explant selection. In vitro establishment is typically better suited with juvenile actively growing shoots resulting from forced flushing techniques, where dormant stems are encouraged to produce flush under controlled conditions (Choi et al. 2014). As in nature, rejuvenation techniques like drastic pruning or rejuvenation grafting to juvenile rootstocks can restore explants derived from mature woody perennials where explants lose morphogenetic potential with the plants age (Dewitte et al., 2003). 'The physiological

state of the source plant has a major impact on the performance of the explant in culture. The regenerative capacity of harvested tissues is determined by factors including but not limited to cell type, nutritional status, water relations, light exposure, and endogenous hormone levels. Explants retrieved from periods of active growth typically yield higher establishment rates and is initial growth vigor than those taken from dormancy intervals. The optimal gathering time varies considerably among species and may be affected by certain climatic conditions or cultural practices. Each species or cultivar of interest inherently requires empirical determination of the most favorable timing of explant collection given this variability.

An additional phenomenon that is recognized as topophysis is described by the fact that the position of an explant on the source plant impacted its performance in vitro. In numerous plant species, nodal segments taken from separate locations on the stem display differential regenerative capability, which is often associated with gradients in endogenous hormone levels, nutrient status, or tissue state. In general, stem explants perform best when taken from the middle portions of the stem and less so when taken from the basal or apical portions of the stem (although exceptions are frequent). Same as the origin, the age of the shoot where explants are isolated affects their reactivity, being the most responsive explants those isolated from younger shoots, which are less differentiated and presents higher activity in meristematic area. Where explant source may be limited (for example high-status genotypes or target breeding lines), alternative measures have been taken to maximize the extraction of valuable material. Microcuttings provide the opportunity to produce multiple explants from source material due to them being tiny nodal segments or shoot tips as small as 1–2 mm long. In such extreme cases, 0.1-0.5 mm tip meristems consisting of the apical dome and a few leaf primordia can be excised and cultured to obtain virus-free plants or to rescue valuable germplasm. These techniques demand advanced technical skills and specialized equipment, such as stereomicroscopes, but can be priceless for propagating rare or hard-to-reach plant materials. The success of axillary bud culture is greatly influenced by the health of the source plants. Although surface sterilization procedures can effectively eliminate external contaminants, systemic infections by viruses, bacteria or fungi may limit explant viability and development in culture. Consequently, practical quality control measures, including the use of disease-free

source plants in stringent screening and selection, are an integral part of quality control in the micropropagation system. For pathogen-free material, approaches such as thermotherapy (exposure of the source plants to high temperature) or chemotherapy (exposition to antiviral compounds) can be used to reduce or eliminate infections in the source plants before collecting explants. Each plant taxa has its specific characteristics and consequently its own optimal type of explant has been identified. Herbaceous species tend to have a broad range of explant sources, ranging from nodal segments to shoot tips, and even leaf axils that contain undifferentiated buds are suitable starting materials. In contrast, woody species typically demand dredgier weep shit for explant, with juvenile tissues (epicormic shoots, stump sprouts and other root sucker) used for woody explant more responsive than mature branch tissue. Because monocot species have a different growth architecture, they are often reliant on various explant sources from their structural morphology (e.g., culm, internode, upper layer of leaf primordia and/or axillary bud, rhizome segments, bulb scales, and/or basal meristems for axillary bud culture). Recent developments in plant biotechnology have added a new battery of tools for the manipulation and improvement of explants. Enhancement of explant responsiveness involves either modulating hormone levels or cellular competency for regeneration via pre-treatments, such dark incubation, cold treatment, and plant growth regulators. In addition, you can design and synthesize novel surface sterilization This enables further adaptation and expansion of axillary bud culture, which is already generalized over various species and multiple propagation contexts, to be integrated with conventional explant selection criteria. In the commercial setting of micropropagation, these factors must be considered with respect to explant sourcing and include intellectual property rights, phytosanitary regulations and logistical constraints. In line with the restrictions for import and export of terrestrial species and for the exchange of material among different countries due to the quarantine regulations to prevent the spread of pests and diseases, international germplasm exchange between countries often involves specialized documentation and testing procedures. These considerations will inform the approaches that are used to expant material for commercial micropropagation practices, alongside practicalities (e.g. distance that the explants need to be transported, conditions to ensure appropriate storage).

Application of Axillary Bud Culture

Axillary bud culture has great potential and broad applications in the fields of plant science and industry, representing a potential technology for basic research and large-scale production of plant material for commercial uses. Its ability to produce large numbers of genetically uniform crops quickly has revolutionized propagation systems for a range of plant species, especially those which are difficult or slow to propagate by conventional methods. This wide applicability is emblematic of the deep biological conservativeness of axillary bud development through the plant kingdom, although species-specific optimizations need to be made for maximal efficacy. The innovative application of axillary bud culture has revolutionised the production industry of many ornamental species in commercial horticulture. The in-vitro methodologies now can be used for the propagation of flowering plants such as orchids, anthuriums, gerberas and chrysanthemums year-round, independent of flowering seasons. This approach allows for rapid propagation of stock for new cultivars, shorter time-to-market for new varieties, and production levels that would otherwise be impossible via traditional cutting or division.). Axillary bud culture has helped to increase the limited natural propagation rate of these plants and reduce their market price with a very fast production process, meaning that for example orchid production is no longer limited to the pace at which they grow in the wild and reduces the price by a great deal and causes the global market to spread. In the same way, foliage crops, bulbous ornamentals, and landscape shrubs reaped the benefits of the faster multiplication rates and space efficiency this technique allowed, with production cycles often shortened from years to months. Axillary bud culture has been valuable for forestry applications resulting in the clonal propagation of elite genotypes with desirable growth, wood quality and/or biotic and abiotic stress resistance traits. This axillary bud multiplication method is now used to propagate many species widely used for plantation establishments including eucalyptus, pine, teak, and poplar, and provides significant advantages over seedling-derived stock including greater uniformity of growth and predictable performance. This technique has been particularly useful for species with recalcitrant seeds, long juvenile phase, or complicated reproductive biology that restricts traditional breeding advances. Moreover, in vitro systems can be managed efficiently in terms of space and resources to preserve various germplasm collections, which in turn support breeding and selection programs for newly developed cultured varieties

that fulfill the growing market needs and environmental situations. The use of axillary bud culture for the improvement of fruit crops has provided an effective approach for newly developed cultivars and rootstocks with improved disease resistance, tissue cultureability, fruit quality or environmental adaptation to be quickly introduced and disseminated. Temperate fruits like apples, pears and cherries and tropical and subtropical species like banana, pineapple, and citrus are now widely propagated using this method. The banana industry has remained especially impacted, as micropropagation through axillary bud culture allows the mass propagation of disease-free plants to support the world-wide trade in this staple plant. The technique additionally enables germplasm to be more easily traded internationally by (1) reducing the volume of material to be moved, and (2) lowering the risk of phytosanitary issues from soil-borne pathogens or soil-borne pests that are likely to accompany conventional propagules. Community conservation of endangered plants is yet another important application area for axillary bud culture to conserve and propagate rare germplasm in case of failure of other conservation efforts. For wild populations that have severely declined, or for species that produce few seeds or are subject to habitat loss, axillary bud culture in vitro in addition offers a means to produce sufficient numbers of plants for reintroductions or ex situ conservation collections. This has been successfully used for many threatened species from a wide range of taxa, including rare orchids/ cycads, endangered trees and medicinal plants. Importantly, the process similarly aids the retention of genotypic diversity within rare genotypes through the maintenance of living stores that conserve the genetic breadth of the species, lending itself to the relative complement of seed banking that can be constrained when faced with seed recalcitrance or viability challenges.

Axillary bud culture has recently been utilized for medicinal plant production to overcome supply constraints regarding species of pharmaceutical importance. Many medicinal plants are slow-growing and/or difficult to culture in the conventional sense, or there is overharvesting in the wild populations which hinders the supply chain for natural product-based medicines. Axillary bud culture by micropropagation serves as a sustainable alternative production system, releasing pressure on wild resources, while also providing consistently high product quality through genetic uniformity. Plants like Aloe vera, Stevia rebaudiana, Withania somnifera, and several traditional Chinese

medicinal plants are now commercially cultivated via this method to support the rising global industry for plant-derived therapeutic compounds and nutraceuticals. One of the most economically relevant uses of axillary bud culture is the production of virus-free planting material. Viral infections in vegetatively propagated crops can lead to significant yield loss and quality reduction, but are not amenable to chemical control methods. Through culture of meristematic tissues (which for some viruses are virus free within infected plants) and extensive indexing for the presence of pathogens, axillary bud culture has been used to develop clean stock programs that supply virus free planting material to growers. This strategy has been particularly effective for continuous making crops, such as strawberries, potatoes, garlic, and stone fruits, in which viral infections, after multiple vegetative propagation cycles, can accumulate and fail to cause the most comfortable gradual degeneration of crops. Axillary bud culture combined with other bio-technological methods gives it the advantage of use for multiple applications beyond mere clonal propagation. Coupled with genetic transformation systems, this method provides a rapid propagation of transgenic lines that can produce enough material for evaluation and eventual commercial use. Likewise, it opens the way for mutagenesis, or selection of somaclonal variation, or induction of polyploids to establish new plant variants with superior properties. This approach also is beneficial for functional genomics studies, as it creates a homogeneous plant material for experimental manipulations, minimizing the confounding effects of genetic diversity that might hinder the interpretation of phenotypic responses. Automated and high-throughput axillary bud culture systems have been developed on a commercial scale to cater for demand, moving from the labor-intensive manual workflow to semi-automated or fully automated protocols that improve throughput and lower production costs. Bioreactor systems have many advantages over solid medium cultures in terms of utilization of space, distribution of medium, and gas exchange, with temporary immersion systems roller bottles being widely used to periodically immerse the cultures in a liquid medium. Multiply rates several times greater than that of their predecessors can be achieved through automation, which also reduces labor needs and contamination potential from fewer transfers.

The use of robotics for explant excision, transfer, and harvesting further streamlines the production process; however, the large upfront capital investment needed makes

it more likely that the technology will be used only in large-scale operations. Economic factors relating to axillary bud culture implementation are greatly dependent on the species used, scale of production, labor costs, and market value of the final product. In high-value crops, such as orchids, berry fruits, or elite tree varieties, micropropagation can incur relatively high per-unit production costs; however, the return on investment is usually sufficient to cover micropropagation costs. On the other hand, for less economically important agronomic crops, axillary bud culture may only be economically justified for limited purposes, for example the rapid multiplication of new varieties or production of disease-free stock plants to be propagated by conventional means thereafter. This economy of scale is still a powerful motivator for invention within production systems, and we have yet to see the full impact of attempts to drive costs lower through process improvements, automation, lean best practices, and economies of scale. Quality management systems for axillary bud-derived plants were adapted from the specific challenges faced in micropropagation, namely genetic fidelity verification, pathogen indexing, and performance evaluation in field conditions. SSRs (Simple Sequence Repeats) or AFLPs (Amplified Fragment Length Polymorphisms) are molecular markers useful to detect genetic variations which could appear during the culture process and PCR-based diagnostics are sensitive method suitable for the detection of viral, bacterial or fungal contaminations. Evaluations (growth, phenology, yield, and stress responses) in post-production field trials are performed to guarantee that micropropagated plants function under commercial growing conditions as planned. While these quality assurance measures might seem expensive, they are vital in order to maintain customer confidence and to support higher prices of micropropagated plants. As new technologies and biological insights emerge, the future of axillary bud culture applications is ever-expanding. Increasing knowledge of the molecular mechanisms regulating axillary bud dormancy and outgrowth should enable more precise manipulation of the multiplication process and result in improved efficiency, broader applicability among recalcitrant species. However, integration with emerging technologies such as CRISPR-based genome editing may help create a sustainable framework for more sophisticated genetic improvements, without losing the advantages of clonal propagation. Moreover, with the increasing focus on sustainable agriculture and biodiversity conservation, there are rising demands for the use of axillary buds that could be relevant for crop improvement, germplasm

preservation, and ecological restoration. These advances herald a new era of research activity that is driving the development of this technique and should ensure it continues to be at the heart of plant propagation technology; bridging the gap between laboratory scale innovation and crops grown on a commercial scale, across a range of plant species and production environments.

Unit 07: Shoot-tip and Meristem Culture

Introduction to Shoot-tip and Meristem Culture

Majority of the plants which we see these days, be it horticulture or agriculture, are due effects of Plant tissue culture which lays down the ground for rapid multiplication as well as conservation strategies for endangered species and improved & superior quality products. Out of all the techniques, shoot-tip and meristem culture are two very important techniques that are used extensively in plant tissue culture. These techniques include isolation and cultivation of the apical or axillary meristems of plants: regions that contain actively dividing cells responsible for plant growth and development. Such methods are important because they have the potential to generate disease-free plants, conserve germplasm, enable mass clonal propagation of plants and provide a base for different biotechnological applications such as genetic transformation and cryopreservation. Although shoot-tip and meristem culture were originally developed during the early 20th century, it was Georges Morel's pioneering work in the 1950s and 1960s that established meristem culture as a reliable method of eliminating viruses from dahlias and potatoes. This was a remarkable breakthrough revealing the practical potential of these methods, the use of which would radically shift horticulture and agricultural practices and allow for the production of disease-free planting material that didn't harbor pathogens and could effectively eliminate pathogens from infected plants. These approaches have subsequently been improved and used in various plant species across numerous taxonomic lineages, and are now invaluable tools in contemporary plant science and agricultural biotechnology. Soot-tip culture is the culture of shoot apices, the apical meristem, and some leaf primordia, generally 0.5 to 1.0 mm in size. Contrast this to the culture of the apical meristematic dome, 0.1 to 0.3 mm in size, without any leaf primordia. Indeed, this point is critical especially for the purposes of virus

elimination, as viruses are known to be present in the meristematic dome but not the meristematic dome (Khalaf & Taliwal, 2003), as the vascular connection is not present (Sadaqat et al., 2018) and the division of meristematic cells outinterbalances the replication of the virus (Alførde et al., 2000) The decision on which method to employ ultimately rests on the aim of the culture, the species subcultured, and the technical skills of the personnel. The basic principle that underlies shoot-tip and meristem culture is totipotency—the potential for plant cells to give rise to a whole plant. When provided with favorable conditions, the undifferentiated meristematic cells in shoot tips can differentiate into various plant organs. These cells can multiply and develop into complete plantlets (organogenesis or somatic embryogenesis) when isolated and grown on nutrient media that supply the minerals, vitamins, carbon sources, and plant growth regulators that they require. This ability for regeneration is exploited in applications, including mass propagation and genetic improvement programs. The efficacy of shoot-tip and meristem culture relies on a number of essential variables, such as the genotypic information of the plant; donor plant age and physiological condition; the season of explant collection; culture medium formulation; as well as environmental conditions throughout the culture process. Some species or cultivars within species may require some changes on these parameters to get successful results. This complication underscores the importance of ongoing studies and adjusting protocols for various plants to optimize these techniques. Shoot-tip and meristem culture applications can be unlocked by advanced technologies in biotechnology in recent years. Integrating with techniques like cryopreservation has allowed for long-term germplasm conservation while merging with genetic transformation approaches has led to the generation of transgenic plants with enhanced traits. Moreover, the integration of molecular diagnostic tools has increased the efficiency of eliminating the virus and certifying plants as virus-free. Such advances illustrate not only the dynamic nature of these approaches, but also their ongoing applicability to 21st century challenges in plant science, agriculture and beyond.

Shoot-tip and meristem culture are specialized types of micropropagation that are concerned with the cultivation of apical and axillary meristems (the growing points of plants, characterized by active cell division). The popularity of these techniques has increased as they are able to address some of the limitations of traditional propagation

methods and help to overcome certain challenges in plant improvement and conservation. In a nutshell, meristematic tissues containing actively dividing and relatively undifferentiated cells are isolated and are grown under aseptic conditions on defined nutrient media to develop whole plants. Shoot-tip and meristem culture differ only in the size and type of explant employed. Culture of shoot-tips: It involves isolation and culture of the apical dome along with 2-3 leaf primordia which generally measure between 0.5-1.0 mm. As this explant size has more differentiated tissues that can support initial growth, it leads to greater survival rates and faster development. Meristem culture, on the other hand, specifically pertains to the culture of exclusively the apical meristematic dome devoid of leaf primordia, typically 0.1 to 0.3 mm. Although more difficult to isolate and establish than other types, meristem culture generally provides better virus eradication because most viruses are incapable of infecting the meristematic dome as there is no vascular tissue there and the cells divide rapidly enough to outrun virus replication. These techniques are theoretically based on the concept of plant totipotency first introduced by Gottlieb Haberlandt in 1902. This concept indicates that the entire genetic information required to recreate a complete plant exists in all living plant cells, given appropriate conditions. Shoot tips contain meristematic cells that are totipotent, meaning they can differentiate into different organs of the plant if the right environmental and nutritional factors are provided. This potential for regeneration underlies the range of uses of shoot-tip and meristem culture in plant biotechnology. The establishment of shoot-tip and meristem culture has been marked by several milestones in their historical development. While the science of plant tissue culture began in the early 20th century, it was not until the 1950s and 1960s that major breakthroughs in shoot-tip and meristem culture were developed. Meristem culture allowed Georges Morel to work out how to rid certain plants, especially dahlias and orchids, of viruses and he was the first to actually get rid of them, showing that he was able to produce virus free plants that had practical utility. Later, Murashige and Skoog developed the MS medium in 1962, which produced the first defined nutrient formulation and opened a new era in academia to improve the success of plant tissue culture, such as shoot-tip and meristem culture. These foundational advances set the stage for the application of these techniques in different plant species. The steps involved in shoot-tip and meristem culture are usually

performed in sequential steps. The initial step involves the choosing and readiness of donor plants — preferably healthy, vigorous, and in a stage of active growth. The second stage is the aseptic isolation of the shoot-tip or meristem under a stereomicroscope, which is challenging and demands a high level of skill and precision. Induction of the initiation medium with the isolated explant, specific nutrients and plant growth regulators are used for growth and development. Then comes multiplication, where we subculture the developing shoots, and rooting, where we move individual shoots to a rooting medium to induce roots. In final phase, adaptation of the regenerated plantlets gradually to ex vitro conditions in order to be ready for field planting.

The nutrient composition of the culture medium has a significant impact on the success of shoot-tip and meristem culture. Commonly, the medium is rich in macro and micronutrients, vitamins, carbon sources (commonly in the form of sucrose), organic compounds, and plant growth regulators. Plant growth regulators have a specific and strong effect in guiding morphogenetic responses of the cultured tissues, according to their type and concentration applied. Common cytokinins include 6-benzylaminopurine (BAP) or kinetin that induce shoot proliferation. Auxins such as indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) are widely used to induce roots. This requirement might vary with the type and genotype of plant, and with the culture phase. The success of shoot-tip and meristem culture is strongly influenced by environmental conditions in culture as well. Some environmental factors that must be tailored include light intensity, photoperiod, temperature, and relative humidity, based on plant species and culture stage. Authored by: Cultures are kept typically at 22–28°C with a photoperiod of 16 h light and 8 h darkness. Cool white fluorescent tubes generally provide an intensity of light from 2000-5000 lux. Which means keeping all the environments right to ensure healthy growth and development of the cultured tissues. FeedBack: Instead of wasting your time with sensory or silly you could prepare your notes and concepts which is great challenge for this test. Isolation of minute meristems (especially for meristem culture) involves inherent technical difficulties and expensive equipment, and the personnel must have good technical skills. a major loss due to the contamination risk especially in isolation and the first culture stages. Furthermore, few species of plants are recalcitrant to tissue culture and require full optimization of the protocols. Other challenges may lie in somaclonal variation, which refers to genetic

changes that can occur during the in vitro culture process, particularly in the case of long-term cultures or when maintaining genetic fidelity is of utmost importance. Moreover, the establishment and maintenance of tissue culture facilities could also be expensive, restricting the widespread application of these techniques, particularly in resource-limited environments. Shoo·t·tip and meristem culture interviewed for this review are only a few of the many biotechnological improvements achieved in relatively recent times that issued biotechnology's avenues and applications. The fact that molecular techniques can detect and eliminate certain pathogens led to their incorporation into micropropagation, and the development of temporary immersion systems and bioreactors improved the efficiency and scalability of the process. Moreover, the combination of these approaches along with genetic transformation methods has led to generation of transgenic plants with enhanced traits. Such innovations underscore the nature of shoot-tip and meristem culture as dynamic, malleable techniques that remain pertinent to the biology and agriculture of today.

Explant Sources for Shoot-tip and Meristem Culture

The choice of explant source is the most important factor that determines the success of shoot-tip and meristem culture. The establishment, growth, and subsequent development of a culture are greatly influenced by the quality, physiological state, and genetic composition of the donor material. The choice of explants sources must be based on several considerations to achieve appropriate responses for these specialized micropropagation techniques. Physiological status of donor plant is of utmost importance in shoot-tip and meristem culture. For most species, explants isolated from actively growing tissue (e.g., spring and early summer) sprout more vigorously. This is due to increased metabolic activity and cell division within meristematic regions in this period. In contrast, explants from dormant or senescent plants may have lower viability and regenerative potential. Thus, harvesting explants when the donor plant is in the phase of active growth can considerably improve culture establishment rates. Similarly, donor plant age affects explant quality in shoot-tip and meristem culture. Explants derived from juvenile or juvenile portions of mature plants generally have a higher regenerative capacity than a mature tissue. The degree of plant secondary metabolite production during the juvenile-mature gradient (also called phase change)

that is likely a result of the cumulative impact of aging on plant tissues: reduced metabolic activity, increased differentiation, and accumulated secondary metabolites. This is especially true for woody perennials, where juvenile materials like seedlings, basal sprouts or rejuvenated tissues need to be used to establish the cultures. Different approaches to rejuvenation, such as vigorous pruning, grafting onto juvenile rootstock plant material, and the use of plant growth regulators can be implemented to pseudo-rejuvenate mature tissues and enhance their responsiveness to in vitro culturing (Botu et al. 2023). Another important element in the quality of the explants is the health status of the donor plant. Derivation of cultures from disease, pest and physiologically normal donor plants for tissue culture is ideal, as the cultures will be free from pathogens and more vigorous. Especially under systemic pathogen infections, like virus, bacteria and fungi, explant from infected plants can either not establish in culture or show abnormal growth in culture. In addition, these infections can remain in the regenerated plants, thereby weakening the final product quality. Thus, donor plants should be regularly monitored and maintained under optimal growing conditions (nutrition, irrigation, and pest management) to ensure explant quality and health. Plant genotypes play an important role in the response to these shoot-tip and meristem cultures. Variability is common between species, and also among cultivars or plant varieties from the same species. This genotypic variability further requires that the culture protocols need to be improved for particular plant materials to obtain reliable and gratifying results. For some species or genotypes, considerable changes in medium composition, growth regulators, and culture conditions must be made to achieve and maintain growth.

The anatomical and morphological type of the donor plant also affect the selection and isolation of explants for shoot-tip and meristem culture. In comparison, simple plants with easily distinguishable apical and axillary meristems are easier to pinpoint explants compared to plants with complex or hidden meristematic regions. In the same way, dehydration of plate cultures of plants with high amounts of trichomes, latex, or mucilage may also complicate sterilization and isolation procedures and require extra steps or changes in protocols to compensate for these barriers. The well-documented seasonal influence on the quality and responsiveness of explants applies to shoot-tip and meristem culture. In temperate regions, explants harvested in the

active growing season (spring and early summer) usually show better in vitro performance and higher establishment rates than those harvested during dormancy (late autumn and winter). This seasonal factor has been explained by changes in the endogenous hormonal balance, nutrient reserves, or metabolic rate of plants in different seasons. Knowledge of these seasonal cues guides the timing of explant collection for improved culture establishment success. Application of pre-treatment of donor plants has a very significant effect on the quality of explants for shoot-tip and meristem culture. The use of growth chambers or/and greenhouses for growing donor plants enables manipulation of environmental factors that can determine the optimal physiological state of these plants. Applying antifungal or antibacterial treatments as pre-treatments can come to reduce the microbial burden on the surface of the plant and make the sterilization steps easier. Telang et al. further reported that explants from certain plants are responsive to in vitro conditions based on physiological changes induced by pretreating the donor plants under certain light regimes, temperature or through the application of plant growth regulators. Another determination of the regenerative capacity of the explant is the placement of the explant in the donor plant. A number of plant species show a gradient of regenerative capacity along the plant axis, with apical parts often outperforming basal ones. The basis of this positional effect is differences in the endogenous hormonal balance, the nutritional status, and the degree of differentiation in different parts of the plant. Spatially dependent effects which also may play a role in the outcome when selecting donor sources of explant to obtain desired cultures. Explants can suffer different storage and transport conditions after collection and during culture establishment that can affect their viability and performance in breeding. The physiological integrity of explants tends to degrade with time in adverse growth environments (e.g., high temperatures, drying, darkness), negatively impacting establishment rates and regeneration potential. Hence, the time lapse from collection to culture and maintaining suitable temperature and humidity conditions during transportation are critical for the quality of explants, especially in the case of sensitive meristematic tissues. Shoot-tip and meristem culture with virus elimination propensity necessitate special considerations when choosing explant sources. As virus load tends to decrease with distance from the protoxylem and therefore from the apical meristem, the smallest possible meristematic dome is chosen

in order to maximize the chance of virus-free cultures being derived. Pre-explant heat therapy of donor plants can reduce virus titer, and thereby further promote virus elimination efficiency. This combination is termed thermotherapy-meristem culture and has been very successful for the production of virusfree plants for a number of crop species.

Application of Shoot-tip and Meristem Culture

Shoot-tip and meristem culture methods have widespread applications in all areas of plant science, agriculture, horticulture and conservation. These methods are versatile and resolve unique challenges in plant propagation, improvement, preservation and significantly contribute to this research arena. These techniques have wide ranging applications highlighting their significance in contemporary plant biotechnology. Shoot-tip and meristem culture is mainly used for the purification of plants infected with viral, bacterial and fungal pathogens. This is especially useful in vegetative propagated crops which include potato, sweet potato, cassava, banana and many ornamental plants where multiplication by conventional means reinfects this pathology and increases it in successive generations. The free vascular connection and the nature of rapid cell division occurring in this meristematic dome makes it a unique niche (often uninfected by viruses) even while the rest of the plant is under systemic infection. It is then possible to regenerate healthy plants by isolating and culturing this virus-free meristematic tissue. For stubborn viruses, thermotherapy (growing donor plants at high temperature (35-40°C) for weeks) or chemotherapy (antiviral compound) in combination with meristem culture undoubtedly can increase virus recovery success. This has played a pivotal role in developing virus-free germplasm collections and certification programs for multiple crops, leading to significant yield gains and economic benefits globally. Another major application of shoot-tip and meristem culture is the mass propagation of elite plant genotypes. However, traditional methods of vegetative propagation are incumbered by low multiplication rates, seasonality and the risk of disease transmission. In contrast, controlled, disease-free conditions in shoot-tip or meristem culture are conducive to rapid multiplication year-round and under replicate conditions of selected genotypes. This high multiplication rate can be obtained by managing the culture medium and in particular the type and concentration of growth regulators which induces multiple shoot formation from a single

explant. This has especially proven valuable in the case of commercial micropropagation of ornamental plants, fruit trees, timber species, and plantation crops. For example, shoot-tip culture for rapid multiplication of disease-free planting material has revolutionized the banana industry and has facilitated setting up of large-scale plantations with highly homogeneous plants. In the same way, all commercial production of ornamentals (like orchids, ferns, and foliage plants) is provided by these techniques to catering to market demands. Plant genetic resource conservation via shoot-tip and meristem culture is essential for recalcitrant seed species, vegetatively propagated crops, and endangered plant species. These include minimising the amount of space required, protection from environmental and pest threats, and controlled conditions that enable long-term storage. Conservation in the short to medium term can be accomplished through a reduction in the rate of culture growth through formulation of culture medium (decreased nutrients or sugar contents), low temperatures, or use of growth retardants. To preserve them long-term, the shoot tips can be cryopreserved in liquid nitrogen (-196°C), which instantly halts cellular metabolism, and indefinitely preserves the genetic material. Through this method applied to several thousands of crop species and their wild relatives, in vitro genebanks have been established to act alongside and complement the conventional seeds storage facilities. These conservation efforts are especially important within the growing threats to the diversity of plant genetic resources caused by climate change and habitat loss, both of which are impacting the availability of crop improvement programs moving into the future.

Shoot-tip and meristem culture techniques have also helped facilitate international germplasm exchange. Plants produced by this methods may be certified free of diseases and thus meet phytosanitary regulations for international transportation of plant materials. Furthermore, in vitro cultures take up comparatively small amounts of space, and grow in controlled sterile environments, decreasing the risk to spreading pests and diseases to new areas. Global research centres—Consultative Group on International Agricultural Research (CGIAR) partners, for example—frequently use these practices and guidelines for the safe dissemination of superior germplasm to collaborators across the globe. Potentially useful plant genetic resources can then be transferred on a one-on-one basis between researchers, leading to rapid introductions of prospective, improved lines into crop improvement programs in the world, having a particularly

strong effect in developing countries, where timely access to elite cultivars significantly boosts agricultural performance and hence mediates food security. We would like to see much more analysis of these diverged populations and even biotechnological applications of them, such as the development of shoot-tip and meristem culture as a basis for genetic transformation and molecular breeding. The ability of meristematic tissues to regenerate makes them excellent candidates for genetic transformation via techniques like Agrobacterium-mediated transformation or particle bombardment. The transformed tissues can then de-differentiate and regenerated to whole transgenic plants expressing the introduced genes. Such approaches have been essential for breeding improved genotypes with desirable characteristics like stress resistance, disease tolerance, and higher nutritional value. Also, shoot-tip culture can be combined with in vitro induction of mutation, selection of somaclonal variation, or haploid production, to increase the amount of variability available for use in crop improvement schemes. Their integration connects classical breeding and modern biotechnology, expediting the breeding of superior plant varieties for enhanced plant improvement solutions. Production of secondary metabolites for commercial application is an emerging use of the shoot-tip/meristem culture. Plants produce a plethora of valuable compounds, including alkaloids, terpenoids, flavonoids, and other phytochemicals that have important medicinal, aromatic, and industrial applications. These compounds can be cultivated under controlled conditions in so-called shoot cultures derived from these plants, removing constraints of seasonality or conditions of the environment. Hydroponics of medicinal plants was discussed, along with the composition of the culture medium that could be modified especially the plant growth regulators to improve the synthesis and accumulation of target compounds. The economic viability of this process can be achieved by scaling up production using bioreactors or temporary immersion systems for high-value products. This is especially critical for endangered medicinal plants, whose survival is threatened from wild harvesting. This being said, there are commercial applications that have used these technologies successfully, such as the production of taxol, artemisinin, and diverse aromatic oils, which showcases the applicative practicality and economic viability of these technologies. Shoot-tip and meristem culture techniques have greatly aided in the understanding of the plant developmental processes. Researchers can isolate and study specific factors in a

standardized, in vitro system that alters and optimizes meristem organization, differentiation, and morphogenesis. The introduction of specific plant growth regulators into the culture conditions should allow one to trigger the desired pathways and result in observable morphological and biochemical markers. These studies shed light on the hormonal control of plant development, the molecular basis of organogenesis and some factors affecting the in vitro recalcitrant nature of some species. In addition, the phenomenon of somaclonal variation, which describes genetic and epigenetic changes that take place during in vitro culture has been extensively studied using these systems and has also greatly advanced our understanding of genome stability and plasticity in plants. These foundational discoveries contribute toward plant biology, as well as feed into the improvement of tissue culture protocols in the practical realm.

The commercial success of shoot-tip and meristem culture is now landmarked by the world plant tissue culture industry with millions of plants formed each year for the agricultural, horticultural, forestry and pharmaceutical sectors. Economic advantages arise due to the fast multiplication factor of elite genotypes, year-round production regardless of seasonal availability, disease-free planting materials, and space and resource efficiency. Commercial micropropagation facilities are now established globally, both at local serving level and large scale facilities that supply international clients. The industry uses many technologies to improve efficiency and cut costs: from automation of culture processes, to temporary immersion systems, to LED lights and disposable culture vessels. While the adoption of these technologies comes with an initial cost, the resulting economic benefits in the form of increased yield, quality and access to the market serve to justify their uptake, especially for high-value crops. There are examples where the adoption of these technologies, such as the banana industry in a number of countries where the transformation of this sector was generated by the use of planting materials produced by tissue culture, have had a significant economic impact on agricultural production and rural livelihoods. While these are just a sample of the many different applications and advantages of shoot-tip & meristem culture, there are a number of specific challenges associated with the cultures that limit their broader adoption and impact. Technical challenges involve isolation of minute meristems, particularly for small-seeded crops or complex plant structures; this task often demands specialized equipment and trained personnel. Practical obstacles –

both biological and practical – stem from the genotypic response of plants to the in vitro environment, with some species or genotypes demonstrating a lack of amenability to tissue culture and requiring the comprehensive optimization of protocols. Economic states: It is often expensive to set up and maintain tissue culture facilities, especially in resource-strapped countries; it limits access to small-scale farmers or businesses. Moreover, prolonged in vitro culturing may lead to somaclonal variation that can compromise genetic fidelity; this is particularly detrimental for applications requiring clonal uniformity. Integrative approaches are needed that combine scientific innovation, capacity building and policy support to make these technologies more widely accessible and impactful across diverse settings. Shoot-tip and meristem culture techniques have developed in recent years and their applications have expanded due to advances in biotechnology. Application of molecular techniques (PCR, ELISA, next-generation sequencing) has enabled their detection and elimination, thus improving virus indexing and certification programs. Advances in cryopreservation protocols, especially with vitrification-based techniques, have greatly facilitated the long-term conservation of plant genetic resources and thus, this technology is now more accessible to resource-limited genebanks. Labor costs in multiplication could be reduced by using systems like temporary immersion systems and bioreactors which lead to efficient and scalable approaches for micropropagation. Moreover, projects involving shoot-tip culture combined with genome editing technologies like CRISPR-Cas9 might hold new promises for crop genome editing. These advances underscore the vibrant nature of shoot-tip and meristem culture, which remain relevant in addressing diverse modern plant science and agriculture needs. Shoot-tip and meristem culture is applied globally to enhance food security, conserve biodiversity, and stimulate economic development. Disease-free planting materials for key crops including potato, sweet potato, cassava, and banana have been produced in quantity, resulting in yield increases in many areas, increased food security, and higher incomes for farmers. The preservation of endangered plant species and crop genetic material through in vitro techniques, which has been important in preserving genetic material for future generations. The establishment of commercial micropropagation industries generated jobs and promoted the local economy. In addition, it has accelerated crop improvement programs worldwide, benefiting all farmers and consumers around the world by facilitating international

germplasm exchange. The multifaceted impacts highlight the transformative potential of shoot-tip and meristem culture in tackling the complex challenges facing agriculture, the environment, and development.

Conclusion

Although shoot-tip and meristem culture have moved from experimental procedures to routine methods in plant biotechnology, from the perspective of their application in plant propagation, genetic improvement, and conservation, they also indicate significant problems still to be solved. Tissue culture techniques, including the ability to regenerate disease-free plants, rapid multiplication of elite genotypes, genetic resources conservation and implementation of different biotechnological applications have positioned these tools at the cutting edge of plant science and cultivation advancements. And, notwithstanding challenges related to technical complexity, genotype specificity, and economic accessibility, innovations in biotechnology are steadily augmenting the usefulness and effectiveness of such approaches. The importance of shoot-tip and meristem culture in creating resilient agricultural systems and conserving plant genetic diversity becomes more relevant as global challenges such as climate change, food security, and biodiversity loss intensify. With the adequate policies and capacity building to support their enhanced application, these techniques can also play an important role in sustainable agriculture and conservation around the world?

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

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

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.

2. 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.

Long 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.
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?

CALLUS CULTURE,

CELL SUSPENSION CULTURE

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.

Unit 08: Production of Haploid Cells - Ovary and Anther Culture

Introduction to Ovary and Anther Culture

Haploid plants are arguably one of the most important aspects in plant biotech have revolutionized the tools available to researchers and breeders to accelerate breeding programs. These haploids, or plants with only one set of chromosomes, have unique advantages for geneticist and breeders alike. The use of ovary and anther culture to produce haploid cells has transformed plant breeding by significantly shortening the time needed to obtain new varieties with the desired traits. Having circumvented the traditional breeding cycles that involve multiple generations of crossing and selection, these techniques allow for the rapid generation of homozygous lines and the emergence and recognition of recessive traits that would otherwise be masked in heterozygous diploid plants. Anther and ovary culture methods are based on the concept of haploid embryogenesis, where the male (microspores) or female (megaspores) gametophytic cells are induced to divert from their endogenous gametophytic developmental route to reach an embryogenic course, resulting in the development of haploid embryos that eventually produce haploid plants. This developmental switch, termed androgenesis when manifested in male gametophytes and gynogenesis in female gametophytes, is a striking example of the extraordinary cellular totipotency exhibited by plants. Haploids have been recommended in wild species improvement programs, by Guha and Maheshwari as a theoretical concept during the 1960s, the first report of successful haploid plant production was from Datura innoxia (under anther culture) in 1964, opening a new era in plant biotechnology. Whether or not such techniques work can be extremely context dependent, and therefore rely on a sophisticated understanding of plant reproductive biology, especially the micro- and megasporogenic developmental stages, and the physiologic and molecular controls over switch from gametophytic to

sporophytic development. The timing of explant harvest is critical, since the responsiveness of gametophytic cells to in vitro culture conditions is highly developmental stage-dependent. Microspores are generally the most embryogenically competent in anther culture either shortly before or shortly after first pollen mitosis when they are at the uninucleate stage of development. In ovary culture as well, the optimal gynogenic response is obtained with ovules containing immature embryo sacs at particular stages of their development. Every single aspect of the culture conditions such as the compositions of nutrient media, plant growth regulators, physical factors such as light and temperature together with different stress treatments play a tremendous role in the induction of haploid embryogenesis. Stress treatment (temperature shocks, osmotic stress, and nutrient starvation), has proven to be the most effective in promoting microspore developmental switch implying that stress response may initiate genes that mediate embryogenic development. Haploid induction is also highly dependent on the genotypic background of donor plants, which show a high variation both among and within species. Thus, although haploid production techniques have significantly advanced, there are still many challenges to address such as genotype-dependant responses, low-efficiency protocols in some important crops from an economic point of view, albinism in regenerated plants (especially in cereals where this represents a serious obstacle), as well as the need to double the chromosomes to foster gametes haploid plants. Ongoing research focusing on unraveling molecular mechanisms of haploid embryogenesis and improving protocols optimized for specific crops and genotypes holds the potential to overcome these limitations . Haploid plants have applications outside of classical breeding programs in areas of plant science such as transformation, mutation studies, genomic mapping and functional genomics. Haploid technology combined with the latest genomic tools and breeding strategies will be extremely important to speed up crop improvement in a world with increasing threats like food security, climate change and sustainable agriculture.

Mechanism of Ovary Culture

Ovary culture, or gynogenesis, is an advanced method utilizing the developmental competence of unused female gametophytes for haploid generation. Ovary culture is similar to anther culture, but while the latter specifically targets male gametophytes, ovary culture has been developed in order to induce embryogenic development in

female reproductive parts, namely the ovules containing embryo sacs. Culture of whole ovaries, isolated ovules or even unfertilized egg cells under in vitro conditions that induced the haploid cells to develop into embryos through parthenogenesis. In gynogenesis, the immature flower buds are harvested when the embryo sac is still empty and contains unfertilized egg cells. Explant collection timing is a critical factor as the receptivity of the female gametophytes to in vitro induction varies dramatically depending on their developmental stage. It has been observed that ovaries with mature embryo sacs and well-differentiated egg cells usually exhibit the best gynogenic potential. After surface sterilization of the ovaries or isolated ovules, they are cultured on (semi)solid nutrient media stimuli that contain the nutrients and developmental factors (vitamins and plant growth regulators) needed to initiate the embryogenic development. Gynogenesis is an epigenetic phenomenon that induces a series of physiological, biochemical, and molecular events which change the usual developmental trajectory of the egg cell from a gametophytic path to an embryogenic path. The degree of this switch is determined by nutritional status, hormonal state, stress conditions, and other developmental decisions. Cell division, differentiation, and morphogenesis depend on a carefully balanced cItent of auxins and cytokinins, which comprises a standard components of a typical solid or liquid medium. Finally, treatments under stress conditions like cold pretreatment, heat shock, or osmotic stress also were reported to strengthen gynogenic induction in numerous species, likely due to the activation of stress-response pathways stimulating the expression of genes responsible for the embryogenic development. When gynogenesis occurs at the level of cells, it is defined by repeated mitotic divisions of the egg cell, resulting in a multicellular haploid embryo. This embryogenic pathway is unique to normal embryogenesis with respect to the absence of fertilization and the addition of a genome from the male gamete. Therefore, the embryo produced contains only maternal genetic material, making gynogenesis particularly useful for genetic research on maternal inheritance. There are a series of phases of development in-between the induced egg cell and haploid embryo. For the first step it transforms from its quasidormant phase into a proliferating state with enhanced metabolic processes and turning on the genes responsible for replication and early stages of development. Then, a series of organized cell divisions lead to the formation of a main body plan of the embryo with different structures forming distinct

protoderm, ground meristem and procambium. As the organism grows, the embryo undergoes further embryo differentiation, forming cotyledons, shoot apical meristem, and root apical meristem. In the presence of favorable conditions, these haploid embryos continue to grow and develop, leading to the formation of fully formed haploid plants.

Efficiency of gynogenesis differs greatly between plant species and genotypes, where monocots usually show higher responsiveness compared to dicots. Some of the crops that have achieved a notable success are onion, sugar beet, cucumber, squash, melon and some cereals. Among many crops, onion (Allium cepa) has become a model system for gynogenesis and has higher induction frequencies. Gynogenesis offers potential advantages, but includes a number of challenges which limit its broader use. The main drawback is the overall lower efficiency of haploid induction compared with androgenesis, requiring the culture of large numbers of ovaries or ovules to be able to generate sufficient numbers of putative haploid plants. Furthermore, it is laborious, particularly in the use of single ovules or egg cells as explants, making it a considerable challenge in technical aspects of microdissection and aseptic status . Additionally, like anther culture, gynogenesis is highly genotype-dependent and there is often considerable variation in response between genotypes within a species. Recently, there are several advances on the molecular mechanisms of gynogenesis, which have revealed the genetic regulation of gynogenetic development. Research has shown that genes and signaling pathways related to cell cycle arrest, hormone signaling, stress response, and embryo development play a role in gynogenic embryogenesis initiation and development. This improved molecular knowledge makes new approaches possible to enhance gynogenesis protocols, either using genetics machinery or by optimizing some culture conditions based on species and genotypes requirements. Gynogenesis-derived haploid plants typically undergo chromosome doubling, either naturally, in culture, or after treatment with agents able to double chromosomes (e.g., colchicine or oryzalin). This process gives rise to doubled haploids (DHs) that are homozygous at all loci and are also fertile, allowing them to be utilized immediately in breeding programs. If the doubled haploids have desirable agronomic traits, they can be used directly as new varieties, or as parents in hybrid breeding programs, thus saving time over conventional breeding programs. Accordingly, gynogenesis introduces

some special advantages over anther culture. This not only leaves little opportunity for regeneration to occur from somatic tissues such as anther wall cells, but also guarantees that all the plants that regenerated are indeed of gametophytic origin. Second, gynogenesis can produce both female-determining (Z) and male-determining (W) haploids (only in species with female heterogamety); these are useful for studying sex chromosomes. Third, gynogenesis should be particularly useful in species in which anther culture does not work, or when male sterility inhibits the application of androgenic methods.

Applications of Ovary Culture

As a versatile and promising approach, ovary culture has been widely applied not only in the production of haploid plants, but also as valuable toolbox for many basic and applied breeding methodologies across the bred and basic plant research area to contribute to plant improvement. Haploid plants derived from ovary culture, with their unique genetic constitution and potential for rapid homozygosity via chromosome doubling, have provided new insights into tackling several agricultural issues as well as basic advances in plant genetics and development. Doubled haploids (DHs) derived from ovary culture have transformed the conventional of plant breeding pipelines by greatly shortening the timeline for producing homozygous lines. Homozygosity gains from selfing using traditional breeding methods usually need six to eight generations to reach reasonable levels, which for any given crop life cycle can range to several years. In contrast, the introduction of DHs into a breeding program can generate perfectly homozygous lines in a single generation, effectively shortening breeding time by years. A major importance of this accelerated development of homozygous lines in hybrid breeding programs is that the fast, economical availability of genetically stable and uniform parental lines will lead to effective hybrid seed production. Additionally, fully homozygous DHs enable a better estimation of genetic merit and combining ability, which in turn allows for the identification of the elite parents combinations for hybrid development. Ovary culture can also be used in genetic studies, allowing features that can be investigated in a very unique manner. Regenerated plants are haploid, facilitating the direct detection and analysis of recessive traits hidden in heterozygous diploid plants. This is advantageous for mutation research, as a recessive mutation

either induced or naturally occurring can be immediately expressed and identified in haploid plants without test crosses or segregation analysis. Moreover, DHs from heterozygous donor plants can give rise to a population of genetically-different homozygous lines that are genetically distinct combinations of the alleles present in parents. These DH populations provide permanent resources for genetic mapping studies by constructing high-density genetic maps, new QTL and marker-assisted selection. DHs derived from ovary culture could have advantages over different mapping populations in functional genomics and gene mapping. DH lines have a fixed genetic constitution, which avoids the complications associated with dominance effects and heterozygosity that can hinder estimating genetic parameters and gene effects. Moreover, DH populations can be replicated infinitely via seed propagation, thus offering renewable genetic resources for multi-environment testing and collaborative research. This has rendered these features indispensable tools in the dissection of complex traits, the identification of candidate genes and the unraveling of molecular markers associating with agronomically-relevant traits. Ovary culture, on the other hand, is integrated with molecular breeding approaches having extensive applications as well in the field of crop improvement. Dilated heterozygotes (DHs) are therefore excellent material for MAS, the interpretability of molecular marker data is simplified and selection can be performed with greater efficiency when not compromised by heterozygosity. In addition, DHs can be directly utilized in the genomic selection programs in which genome-wide markers construct the predictive model to determine the breeding value of individuals according to their genetic backgrounds. DH lines, due to their genetic homogeneity and stability, may also improve the accuracy of genomic prediction models, ultimately leading to better-informed breeding decisions and faster genetic gain. Ovary culture plays an important role in the efficient selection and fixation of induced mutations in mutation breeding. Both donor plants and cultured ovaries can be mutagenically treated, enabling the recovery of haploid plants. The haploid condition enables immediate visibility of recessive mutations, or alternatively results in subsequent chromosome doubling fixing those mutations in a homozygous manner. This strategy has been effectively used for creating crop varieties with desirable features, including disease resistance, stress tolerance, and quality traits.

Lastly, for polyploid breeding and interspecific hybridization, cladistic studies of ovary culture and bestow new characters of ovary structure will cohere a novel kaleidoscope against the low productivity of breeding by, for example, inflicting more genetic broadeners of cultivated that found the transgression of gene cultural. The reduction of polyploid species (e.g., by gynogenesis) allows the genetic analysis of polyploids, as well as the possibility of introgressing traits from lower-ploidy relatives. In interspecific hybridization this can be used to generate fertile doubled haploids from the hybrid through gynogenesis, as in normal sexual reproduction, hybrids are often sterile due to chromosomal incompatibilities. It has been especially useful in wide crossing programs, where breeders use sexually incompatibility with wild relatives as a strategy for introducing favorable traits into crops. Ovary culture, combined with genetic transformation technologies, provides valuable tools to improve crops and conduct functional genomics studies. An alternative is to use ovary culture-derived haploid tissues or cells as targets for genetic transformation, followed by chromosome doubling to yield DHs that are homozygous for the gene(s) that is introduced. This strategy eliminates the requirement of multiple generations of selfing to isolate homozygous transgenic lines, speeding up the generation and assessment of transgenic crops tremendously. In addition, the haploid condition offers an ideal genetic context in which to investigate gene function, since the phenotypic effects of imported genes can not be confounded by either dominance or heterozygosity. Ovary culture presents novel strategies for germplasm conservation and rapid propagation in the conservation and use of plant genetic resources. For such wild species or landraces with long life cycle or vegetative propagation, the induction of double haploids (DH) by gynogenesis (producing only female haploids) can speed up the characterization and utilizations of their genetic diversity in breeding programs. Also, this approach could be used to perform ex situ conservation for rare or endangered species as well as to rapidly multiply genetically diverse individuals. Ovary culture has been an important tool in basic research on the reproductive biology, embryogenesis, and totipotency of plant cells, as it has improved our understanding of these fundamental processes. Therefore, studies of gynogenic embryo development help us understand the molecular and cellular mechanisms during embryogenesis, such as how gametophytic development is switched to sporophytic development. Additionally, the study of factors controlling

gynogenesis has provided new insights into cell fate determination and developmental plasticity in plants. Ovary culture, a practical crop improvement strategy has been practiced in several species with significant impact in some economically-important crops. This gynogenetic method has emerged as the method of choice for DH production in onion (Allium cepa), accelerating inbred line development for hybrid breeding and genetic improvement of traits such as disease resistance, bulb quality, and adaptation to various growing environments. Gynogenic haploid individuals (DHs) have proven to be especially relevant for breeding programs in sugar beet (Beta vulgaris), especially in producing monogerm hybrids which exhibit enhanced yield, sugar content, and disease resistance. In cucumber and other cucurbits, gynogenesis has enabled the production of pure lines for hybrid seed production and for the genetic analysis of important horticultural traits. Ovary culture has many applications and successes, yet several challenges and opportunities for further research and improvement remain. The high genotypic dependency of sterility responses is still a major bottleneck to gynogenesis applications in horticulture, with many economically important genera and genotypes being relatively unresponsive to current in vitro exploitation protocols. To overcome this limitation, a detailed understanding of the genetic basis underlying gynogenesis along with the development of crop- and genotype-specific strategies are necessary. Therefore, the low efficiency of gynogenesis, relative to androgenesis, means cultures, pretreatments and combinations of growth regulators should be further optimized to improve induction frequencies and regeneration rates. Genomics, transcriptomics, metabolomics and other molecular biology techniques can be applied to help better clarify the molecular mechanisms of gynogenesis and facilitate the discovery of important genes and signaling pathways related to gynogenesis. This knowledge will enable marker-assisted selection for gynogenic capacity, genetic engineering strategies for improved induction response, and more hone tecnhological generic optimization of culture protocols based on the molecular requirements of target plant species and genotypes. To summarize, ovary culture is a powerful and versatile technology that could facilitate applications in many areas of plant breeding and research. With ongoing improvements of this technique and its combination with modern genomic technologies and breeding methods, it has significant potential for accelerating cropping improvement and advancing our knowledge of plant reproductive biology and development. PDF | The pressures of

population growth in the context of climate change and limited resources are exerting increasing demands on global agriculture, thus making the efficient development of improved crop cultivars by technologies such as ovary culture ever more vital to ensure food security and sustainable agricultural production.

Unit 09: Somaclonal Variations

Introduction to Somaclonal Variation

Somaclonal Variation An Fascinating PhenomenonSomaclonal variation is considered as one of the most interesting phenomenon related to plant tissue culture with both challenges and opportunities for plant biotechnologists and plant breeders. Somaclonal variation was first defined by Larkin and Scowcroft in 1981 and it refers to the genetic and/or phenotypic variation between the regenerants from which the variation has originated from somatic cells by tissue culture. This phenomenon has now spurred a field of research in its own right, from the basic understanding of such genomic plasticity to its application in crop improvement. The phenomenon of somaclonal variation arose from the observation that tissue-culture regenerated plants frequently display characteristics that differ from the donor plant. Somaclonal variation, originally viewed as an undesirable event resulting in reduced genetic fidelity of micropropagated plants, is now accepted as a possible resource to generate new genetic variability. This can lead to differences in morphological traits, yield components, disease resistance, stress tolerance, and biochemical properties among clones. Somaclonal variation: Somaclonal variation is the phenomenon concerned with the genetic differences seen between a plant and its respective tissue culture derived somaclonal plant that is supposed to be genetically identical to the parent plant. This lack of genetic fidelity can be both a restriction and an advantage in plant biotechnology. As one side it raises concerns for certain applications where genetic stability is a top priority, namely germplasm conservation, and commercial micropropagation, as it is the very nature of A. tumefaciens to alter the plant genome that hampers applications of genetic engineering for the production of clonal plant material. On the positive side, it provides a mechanism for creating novel genomic combinations that could be leveraged to improve crops. Somaclonal variation cannot be appreciated without a thorough understanding of the

complex cellular and molecular events that occur during the tissue culture process. In tissue culture, plant cells are subjected to stress from being stripped from the organized context of their tissues, and to artificial conditions that elicit a broad variety of cellular responses. During regeneration, dedifferentiation, followed by redifferentiation, is a process involving considerable reprogramming of cellular pathways, likely resulting in genetic and epigenetic changes. Somaclonal variation occurs across a spectrum that includes everything from changes in ploidy level and chromosomal rearrangements to point mutations, transposable element activation, and epigenetic changes. Some of these changes are random across the genome, while others may be focally enriched. FigurePanelAandBVariability in elicitor production and secretion due to changes in variables such as genotype, explant type, medium composition, and culture period. Somaclonal variation is not only of basic biological interest. Provides practical implications for plant breeding, germplasm improvement and management of genetic resources. Somaclonal variation has been used by plant breeders as a source of new traits for crop improvement, providing numerous commercial varieties with improved traits. Somaclonal variation has also enhanced our understanding of genome organization, stability and evolution in plants. With a focus on somaclonal variation, its mechanisms, drivers and applications, it is clear that it constitutes a fascinating instance of genomic plasticity in plants an extraordinary capability plants have to respond and adapt to changing environments. Somaclonal Variation: Implications in Plant Biology and Breeding The potential of somaclonal variation in crop improvement continues to ignite scientific inquiry and exploration, as new variants are screened for their utility in breeding programs.

Understanding Somaclonal Variation

Somaclonal variation is defined as genetic and phenotypic variation occurring in plants dervied from somatic cells in tissue culture. This phenomenon was also formally recognized and named in a seminal publication by Larkin and Scowcroft in 1981, although observations of unexpected variability in tissue-cultured plants had been noted by researchers 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. Somaclonal variation can be observed in the field with varying of phenotypic effects. Some of the regenerated plants may have deviations in leaf shape or size, growth habit, flower structure or color, fruit traits, or biochemical composition. Such phenotypic changes can be stably heritable via sexual reproduction, implying genetic modification; or they can be transient, implying epigenetic modification that can revert to the original state in subsequent generations. Somaclonal variants frequently exhibit chromosomal changes (in the number and/or structure of chromosomes) from a cytogenetic standpoint. These changes can be aneuploidy (loss or gain of individual chromosomes), polyploidy (multiplication of the full set of chromosomes), chromosomal deletions, duplications, translocations, or inversions. This is typically associated with large phenotypic impacts on plants and often results in sterility or partial sterility when severe, depending on the type and extent of cytogenetic changes. Somaclonal variation is variable depending on several factors. Some species and genotypes of plant are more predisposed than others to show variation as a consequence of tissue culture (somaclonal variation). Certain tissue culture protocols are also known to propagate higher rates of variation than other techniques. It can be detected in up to >30% of regenerated plants or as little as <1%, rendering it a major consideration in tissue culture applications.

It is relevant to distinguish between somaclonal variation and other sources of variability that may also arise during plant regeneration. Somaclonal variation is distinct from spontaneous natural mutations, which occur at low frequencies. It occurs in the absence of intentional exposure to agents designed to damage DNA, unlike the variation induced

by mutagens. In contrast to somatic mutations in intact plants that typically remain restricted to particular tissues or sectors, somaclonal variants arising in regenerated plants can alter the entire organism and can be passed through generations. Diverse methods are utilized for the detection and characterization of somaclonal variants. Morphological traits were the first to be used in phenotypic screening; thus, they are the most straightforward way to track obvious variants. Biochemical analyses, cytogenetic techniques and molecular markers provide more robust approaches to the detection and characterization of changes at the cellular and molecular levels. Recent genomic techniques, specifically next-generation sequencing, enable access to the genetic basis of somaclonal variation with unmatched resolution. Somaclonal variation is an extremely important factor in plant biotechnology and breeding. Somaclonal variation is a concern in commercial micropropagation and germplasm conservation, where it is important to maintain genetic fidelity and not allow this variation to be transferred into cultures eventually propagated in the field. Alternatively, for crop enhancement programs in search of new genetic combinations, somaclonal variation serves as a useful source of novel traits for selection and incorporation into breeding lines. This two-sided nature challenge and opportunity makes somaclonal variation an interesting field of study with important applied consequences. Both of these papers paved the way for more detailed investigations using molecular and genomic tools to understand mechanisms of somaclonal variation, studies which have accumulated in the decades since. Somaclonal variation, first observed as early as 1983, remains an area of active research, with its scope expanding since our understanding of plant genomics and epigenetics has broadened, allowing us to predict, control, and take advantage of somaclonal variation for a wide variety of applications.

Mechanisms of somaclonal variation

Somaclonal variation reflects a multitude of genetic and phenotypic changes from a combination of cellular and molecular mechanisms. Knowledge of these mechanisms contributes to controlling undesired variation while harnessing beneficial changes for crop development. Genetic and epigenetic changes, as well as chromosomal defects, are the main mechanisms responsible for somaclonal variation. Somaclonal variation is attributed to genetic mechanisms as well as changes in the DNA sequence itself. Point mutations are an advanced type of genetic change, and can be a single nucleotide

substituted, inserted or deleted. It can affect all parts of the genome but it generally has more significant impact when affecting the coding regions or regulatory elements of genes. Although the spontaneous mutation rate is generally low in plants, the stress induced by the tissue culture environments seems to significantly increase mutation rates. Proliferation of SNPs in regenerated plants relative to their donor plants are confirmed by advanced sequencing technologies. Gene amplification and deletion events represent another key genetic mechanism. These changes result in changes of copy number of individual genes or segments of the DNA, which may affect levels of expression of genes. The repetitive structure of plant genomes, with large numbers of duplicated genes and transposable elements, makes it relatively easy for copy number variation to occur via mechanisms such as unequal crossing over during DNA replication or repair. Effects of large-scale gene amplifications can be dramatic, especially in the case of genes associated with growth control, stress response or secondary metabolism. A particularly important mechanism of somaclonal variation is transposable element activation. Plant genomes harbor a multitude of mobile genetic elements that can relocate to a new genomic site, either by a cut-and-paste mechanism (transposons) or a copy-and-paste mechanism (retrotransposons). These components are usually silenced in somatic tissues, but can be reactivated under the stress of tissue culture conditions. The transposition of such elements can disrupt gene functionality, modify regulatory networks, bring about chromosomal rearrangements, and produce novel genetic variations. Genome-wide studies of various species confirm that transposable elements are extensively reactivated in tissue culture, consistent with some of the somaclonally variable traits 246,247. Genetic variation can also arise from somatic recombination events during mitosis. While homologous recombination normally takes place in meiotic germ cells, it may also transpire at times in somatic cell division phases, especially during stressed conditions. These recombineering events rearrange genes, creating new ones and/or deleting or messing up other ones. The promiscuity of cell division that accompanies such undifferentiated callus cultures, which often have compromised cell cycle checkpoints, might allow for such recombination events to occur.

In addition to genetic mechanisms, epigenetic variation is an important contributor to somaclonal variation. Epigenetic changes are based on the translation of the genetic

code into active or inactive chromatin to modify gene expression without altering the DNA itself, most significantly, by means of DNA methylation, histone modifications and chromatin remodelling. The epigenetic reprogramming of dedifferentiation and redifferentiation, which occurs during plant regeneration, may not necessarily revert the epigenetic state of the donor plant ([Das et al., 2021]) and involve substantial epigenomic remodeling (Wang et al., 2019, Flanagan et al., 2020). One of the best-established epigenetic mechanisms involved in somaclonal variation is DNA methylation patterns, and modification of cytosine residues is the most prominent form of DNA methylation. In fact, according to tissue culture conditions, specific genomic regions can be both hypermethylated (over methylated) and hypomethylated (down methylated). Changes in methylation status can have meaningful effects on gene expression and thus have effects on phenotypes. Extensive methylation changes have been observed in regenerated plants in genome-wide studies, both in coding sequences and also transposable elements. Importantly, although certain methylation changes are ephemeral, some persist for many generations explaining the patterns of inheritance seen within certain somaclonal variants. Histone modifications provide another level of epigenetic control that can be modified in tissue culture. Such modifications such as methylation, acetylation, phosphorylation and ubiquitination can change chromatin structure and thus accessibility for transcription. The tissue culture induced aberrations in histone modification patterns can render to differential expression profiles of plant regeneration. The dynamic interactions between DNA and histone marks form a complex local regulatory network, which can be altered by the stress of in vitro culture. Small RNAs, such as microRNAs (miRNAs) and small interfering RNAs (siRNAs), are another epigenetic mechanism associated with somaclonal variation. These RNAs are key players in gene regulation and transposable element silencing. The expression patterns of small RNAs can be changed in tissue culture conditions, possibly affecting the gene expression of these and other different genes as well as transposons. Results obtained with high-throughput sequencing approaches have uncovered differential expression of many small RNAs in tissue-cultured cells versus corresponding intact plant tissues and these changes correlate with phenotypic differences seen. Compared to point mutants or epialleles, chromosomal events are more drastic both phenotypically and genetically, and thus play a large role in somaclonal variation. For example, some chromosomal abnormalities arise from errors during mitotic cell-division in tissue-culture, so this type of change can

management of somaclonal variation, either for applied purposes in commercial micropropagation or targeted to enhance the application of somaclonal variation in crop breeding programs.

Factors Influencing Somaclonal Variation

A complex interaction of factors governs the incidence and magnitude of somaclonal variation. These variables may be grouped into plant material variables (genotype, the explant source, and physiological status), culture condition variables (medium composition, growth regulators, and physical environment), and regeneration process (method, duration, and number of cycles) variables. Knowledge of these factors is important for both prediction, and optimization of somaclonal variation for different uses. The tendency for somaclonal variation is a function of genotypic factors. Somaclonal variation has an unpredictable potentiality among different species. Cereals like rice, wheat and maize generally exhibit a higher frequency of somaclonal variation in comparison to legumes or certain vegetables. Even within a single species, different cultivars or genotypes can show strikingly different propensities to variation. This genotypic impact may also be related to variation in genome architecture, DNA repair pathways, chromatin status and transposon content across plant lineages. Somaclonal variation patterns are influenced strongly by the ploidy level of the donor plant. Polyploid species are generally more tolerant of chromosomal aberrations and genetic alteration than their diploid counterparts as a result of the buffering effect of extra gene copies. Such genetic redundancy enables polyploids to tolerate significant genetic changes without lethality, which likely results in increased frequencies of viable somaclonal variants. However, the phenotypic changes may not follow the same patterns and in fact, polyploids often show less dramatic phenotypes, even while experiencing more extreme genetic changes. Genomic size and architecture associate with the propensity for somaclonal variation. Such cases are more frequent for plant genomes with larger size, especially for those characterized by high amounts of repetitive DNA and transposable elements, and point out a clearer susceptibility to somaclonal variation. The high density of repetitive sequences can promote recombination events as well as chromosomal rearrangements and large numbers of transposable elements offer targets for activation in response to tissue culture stress. The quantitative pattern of somaclonal variation at different genomic locations is also influenced by the structural

organization of the genome, in particular the distribution of heterochromatin and euchromatin regions (Bray). The origin and kind of explant utilized for the establishment of cultures are key factors influencing somaclonal variation. Each type of tissue has a different cellular composition, developmental state and epigenetic profile, resulting in differential responses to tissue culture condition. Highly differentiated tissues (leaves, stems, with meristematic self-renewal, as well as roots) require greater dedifferentiation and cellular reprogramming processes during regeneration than those containing meristematic cells and this is likely to elicit more extensive genetic and epigenetic changes.

Differently differentiated tissues produce less somaclonal variation, while meristematic tissues such as shoot apices, axillary buds, or embryonic tissues, produce regenerants with the least somaclonal variation. The stability demonstrates the meristematic cells inherent undifferentiated state, resulting in less severe reprograming requirements for regeneration. Furthermore, meristematic tissues experience active cell division throughout life, with more functional DNA damage repair than differentiated somatic tissues, which may further allow for more effective checkpoint controls in place to regulate active cell division under natural conditions. The somaclonal variation results can be significantly affected by the physiological and health status of the donor plant. However, the cellular function and genomic stability of explant cells will be affected when plants are subjected to stress conditions like drought, nutrient deficiency or pathogen infection. Prime examples of how donor tissue age can modulate variation come from the analysis of either donor age and genetic background, with older tissues tending to exhibit higher mutation rates and epigenetic changes compared to juvenile tissues. Conditions such as these may already exist prior to tissue culture and promote higher somaclonal variation as the plants are regenerated. One of the main categories of factors that affect somaclonal variation is culture conditions. The formulation of the culture medium, specifically the hormonal ratios and concentrations, is a major contributing factor. Consistent up, 4-dichlorophenoxyacetic acid (2,4-D) heights 2,4-D 4-D, principally synthetic auxin, have been proportionate with auxiliary frequencies of somaclonal variation. These synthetic growth regulators may cause changes in DNA methylation, activation of transposable elements, and disturbances of normal cell cycle regulation, which can

contribute to genetic and epigenetic instability. Patterns of somaclonal variation are also determined by the cytokinin type and concentration. Chromosome doubling and also cytogenetic abnormalities were induced by certain cytokinins especially in concentrations100mg/Kg. Depending on the ratio of auxins and cytokinins, which dictate the developmental pathway (the formation of callus, organogenesis, and somatic embryogenesis) in tissue culture, somaclonal variation has nature and extent. Plants obtained through direct regeneration pathways lacking a cytokinin callus stage, in general have lower rates of variation. The nutrient composition and availability in the culture medium also plays a role in cellular metabolism and stress responses. Stress conditions related to high concentrations of certain minerals, especially ammonium ions, were deemed to induce somaclonal variation. Likewise, osmotic conditions and energy metabolism change with sugar type and concentration, and high sucrose concentration is associated with increased variation frequency. This oxidation effect can damage DNA and disrupt molecular function, so the reduction of variation can be improved through antioxidants used in the medium. Various factors contribute to somaclonal variation, and physical culture conditions are perhaps the most important ones involved such as light quality and light intensity, temperature, and gaseous environment and so on (Jin et al. Light conditions can impact the level of photosynthetic activity and photomorphogenic responses, which in turn may include cellular stressors and DNA repair pathways. The integrity of our DNA, which is the basis of our genetic structure, is affected by temperature extremes or fluctuations, which can disrupt normal cellular processes such as DNA replication and chromosome segregation during mitosis. Closed vessels used for culture allow the accumulation of ethylene and other volatile compounds that can constitute additional stress conditions that favor genetic instability. Indirectly, availability of nutrients and cellular functions affected by the pH of the medium can contribute to somaclonal variation. An extreme pH milieu induces cellular stress, which causes higher mutation rates and epigenetic modifications. In addition, pH fluctuations during culture, often due to proliferation, infarctions, or secondary metabolite release, may modify stress conditions over time, altering long-term genomic stability in culture.

Somaclonal variation outcome is greatly affected by the regeneration method used. In contrast to indirect regeneration through callus, direct organogenesis generally results

in lower variation rates in regenerated plants. While somatic embryogenesis, especially direct embryogenesis, tends to produce plants with higher genetic fidelity than organogenesis via callus, this trend is not consistent across species and culture systems. Duration of callus phase is one of the most important factors that controls frequency of somaclonal variation. High-maintenance of cells in persistent undifferentiated callus increases the chance of genetic and epigenetic alterations during consecutive divisions. Dysregulated cell division in callus — which frequently has compromised checkpoint controls — allows the propagation of genetically abnormal cells that would otherwise be eliminated in organized tissues. Several species of plants have been shown to display somaclonal variation and competition with continuous cell cultures at their callus cultures. Subculture cycles vary widely in long-term cultures and are a major determinant for patterns of variation. For each subculture event, a portion of the callus is selected for transfer to fresh medium, and so this process may create genetic bottlenecks, or select cells that have greater growth potential, and may carry genetic or epigenetic alterations. The accumulated stress of multiple wounding with subculture, in addition to exposure to new growth regulators, can also induce further genetic instability. When purity of genetics is a primary concern, minimizing subculture frequency is a common recommendation. Somaclonal variation in the genotype of the culture is mediated by the specific systems to be used in the culture process (solid vs. liquid medium), which would vary in terms of nutrient availability and cellular interactions due to gas exchange effects. Liquid cultures generally ensure a more homogenous exposure of cells to the nutrients and growth regulators, but at the same time lead to hypoxic conditions, which stress the cells. Bioreactor systems designed for large-scale culture generate additional sources of stress such as shear stress or pressure changes that may impact genetic stability. Somaclonal variation is influenced by some special features in cryopreservation-based methods, which have been increasingly applied for germplasm conservation. Cellular stress due to freezing and thawing, as well as exposure to cryoprotectants, can profoundly affect genetic stability. In fact, cryopreservation stops cellular division in storage, which may reduce genetic changes that might otherwise accumulate with continued culture. These parallel effects of cryoinjury and suspended growth result in complex patterns of genetic stability in cryopreserved tissues. Consequently, somaclonal variation, even under stringent experimental controls, is likely to differ across laboratories due to laboratory-specific

factors like handling practices, equipment calibration, and environmental control. Such considerations emphasise the significance of standardised protocols and quality assurance methods, particularly in the case of commercial micropropagation services where genetic fidelity is a prerequisite. Predicting somaclonal variation in a particular culture system is complicated due to the interaction of the various factors. Nonetheless, such complexity provides opportunities to manipulate conditions to either exploit minimum variation (for clonal propagation or germplasm security) or increase variation (for crop enhancement opportunity). Therefore, by comprehending these impacting factors, researchers and practitioners can formulate tissue culture protocols more suited to their specific goals in terms of genetic stability or variability. These influencing factors have practical implications in diverse applications of plant tissue culture. Using small amounts of growth regulators at high concentrations, obvious regeneration pathways, or long culture duration is not typically found in protocols designed for the micropropagation of commercial species where genetic fidelity is critical and the aim is to produce millions of plants. In contrast, degenerating explants, high auxin concentrations, extended callus culture, and numerous regeneration rounds may be purposefully employed in crop improvement programs aiming for new genetic combinations to drive variation. Ongoing investigations in this field are focusing on generating more refined predictive models that incorporate the range of causal factors to predict the probability of eventual somaclonal variation occurring in individual culture systems. These models are expected to improve the efficiency and reliability of multiple tissue culture applications, from commercial propagation to the fundamental study of plant genomic plasticity.

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 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. This strategy is especially valuable for vegetatively propagated crops, long-generational perennials, or highly heterozygous species with significant hurdles to conventional breeding. Many commercial cultivars derived through somaclonal variation were released among different crop species. Varieties such as 'Co 86032' in India and 'Copersucar' series in Brazil were developed from somaclonal variants in sugarcane, which exhibited improved yield and disease resistance. Somaclonal selection programs aiming to improve tuber quality and disease resistance were used to develop potato cultivars such as 'AAFC- Summit Russet' and 'Rochdale Gold-Dorée'. For ornamental crops, many cultivars with new flower colors, patterns, or growth habits were commercialized, especially in chrysanthemum, carnation, and lily. Such commercial successes are evidence of the practical significance of somaclonal variation for the production of improved cultivars for agriculture and horticulture. The development of disease resistance is one of the particularly successful applications of somaclonal variation. Under tissue culture conditions, defence-related genes and pathways may be activated, which may result in increased resistance to a range of pathogens. Because all stages of life are present in vitro, screening for disease resistance can take place directly, by the addition of pathogens or their toxic metabolites in the culture medium, which allows for rapid assays of large populations. The plants regenerated could also be evaluated in the field with or without disease pressure from naturally occurring or artificial sources. Examples include rice varieties with resistance to blast and bacterial blight, potato with resistance to late blight and viruses, sugarcane resistant to eyespot disease and banana resistant to Fusarium wilt. The molecular basis of this resistance usually includes the modified transcriptional programs of the defense-related genes or the altered recognition of pathogen respective factors. Given the rising concern about the effects of climate change on agriculture, improvement of crop abiotic stress tolerance using somaclonal variation has received a good deal of attention. Inner and outside stresses are also an integral part of the tissue culture environment leading stress responses that can be retained upon regeneration of plants.

One approach to discovering species or variants with improved tolerance is to perform in vitro selection using agent the stresses to which the plant is adapted such as salt, compounds that mimic drought (polyethylene glycol), heavy metals or extremes of temperature. Among the examples of notable successes are rice varieties that were developed for enhanced tolerance to salt and drought, wheat varieties with improved aluminum tolerance, and sugarcane with improved water-use efficiency. They exhibit a despair that could lead to the generation of germplasm with tolerance to abiotic stress for their incorporation into breeding programs, and provide clues to the mechanisms for stress adaptation in all plants. Another important application of somaclonal variation is quality improvement of crop products. Crop plants have also been known to exist in variant forms with alterations in nutritional composition, taste profiles, or processing traits (Bertorelli and Swanson, 2020). In potato, somaclonal variants with altered starch properties, reduced glycoalkaloid content, or improved frying quality could be developed. Commercially successful traits have been identified in tomato, including variants with increased lycopene levels, extended shelf-life, or modified flavor compounds. For medicinal plants, somaclonal variation has given rise to lines with higher production of pharmaceutically important secondary metabolites, including alkaloids, terpenoids, or phenolic compounds, improving their medicinal and economic significance.

Yield improvement using somaclonal variation is economically significant in some crops. Inducing mutations to develop plants with modified architectural features, elevated photosynthetic efficiency, improved nutrient absorption, or have shifted source-sink relations can confer significant yield benefits in comparison to parent cultivars. Somaclonal variants with increased tiller number, panicle size, or grain filling have been created in rice. Variants that are characterized by increased sucrose accumulation or biomass production have enhanced industrial yields in sugarcane. These yield traits can also be due to any genetic or epigenetic change that alters hormonal homeostasis, metabolic pathways and/or developmental trajectory of the plant. One highly useful application of somaclonal variation is to overcome this innate trait and allow crops to colonize new growing regions, a concept which leads to adaptation to distinct environmental conditions. Variants adapted to longer or shorter day lengths, different temperature regimes, or soil types can broaden the range of cultivation of some

economically important crops. Examples include wheat cultivars that flower earlier and tolerate short season environments, heat tolerant subtropical bananas, or rice cultivars that perform better under limited irrigation developed using somaclonal selection. This application is of growing prominence in an age of climate change, where adaptation of crops to new environmental regimes is vital to sustainable agricultural practices. Somaclonal variability is a rationally targeted application for germplasm enhancement and widening genetic diversity in production-based breeding programs. Most modern crop varieties are based on a narrow genetic base, leading to vulnerability to new pests, pathogens and changing environments. Somaclonal variation is capable of offering new genetic combinations to a breeding program, which may break undesirable linkage drags or introduce a trait not found in existing germplasm with relative ease. They might not be directly used as commercial cultivars, but the variants provide important genetic resources for breeding and the options for further diversifying the crop genepools. Somaclonal variation has important scientific applications in the characterization of variants as well as the genetic study of somaclonal variation. Somaclonal variants can exhibit changes in genetic and phenotypic traits that offer clues to genomic structure, gene function and developmental control. Efforts to dissect the molecular basis of individual variant phenotypes can identify genes and regulatory networks that contribute to key traits, therefore further elucidating plant biology. This goal has been expedited by the application of advanced genomic approaches such as whole-genome sequencing, transcriptomics, and epigenomic analyses to accurately characterize the genetic and epigenetic changes that underpin somaclonal phenotypes. Somaclonal variation is a promising tool for mutational breeding and expands the classical genetic toolbox of techniques for mutagenesis. The tissue culture environment per se is mutagenic, provoking a range of genetic changes that are different from what is normally produced by chemical mutagens or radiation. The use of tissue culture in combination with classical mutagenesis will improve the mutation frequencies while generating new genetic malformations. This integrative strategy has been effective in generating better types of numerous crops and can produce more effective and novel variation patterns than either method alone. Polyploid induction constitutes one form of somaclonal variation with important implications for breeding. Polyploidization, an important process in plant breeding, can happen spontaneously during tissue culture conditions and is commonly induced by certain combinations of

growth regulators. Such polyploid plants exhibit traits such as large organs, novel secondary metabolites, or increased resistance to stress that can be of agricultural or ornamental significance. Inductions of controlled polyploids by tissue culture have been especially successful in breeding programs for ornamental crops, where increased size of flowers or altered morphological traits are desirable to commercial growers, and in the production of triploids to create seedless fruits. While not directly somaclonal variation, haploid and doubled haploid production can be considered a closely related application where tissue culture confers genetic changes upon a plant, resulting in potentially useful breeding materials. Haploids can even be produced through the culture of anthers, microspores, or ovules which can then go through spontaneous or induced chromosome doubling to give rise to doubled haploids. These near-isogenic lines are extremely valuable for breeding programs since pure lines can be produced rapidly and genetic systems can be explored (all lines are homozygous). Tissue culture conditions apply stress to the seedlings, which can affect haploid induction efficiency and plant genetic stability, linking valuable haploid induction applications to more general mechanisms of somaclonal variation. Research on somaclonal variation has also played an important role in the development of commercial micropropagation protocols. An understanding of the contributions to genetic stability has resulted in improved protocols to minimize undesired variation. These adjustment factors consist of: use of meristematic explants, using pathways of direct regeneration, optimizing the concentrations of growth regulators, minimizing in vitro culture times, and performing regular monitoring for genetic purity. Refinements in protocols have resulted in more reliable and consistent commercial micropropagation operations, which are especially valuable for high-value crops where uniformity is critical.

Somaclonal variation can affect genetic transformation or genome editing efficiency but also offers a double-edged sword. Tissue culture induces genetic changes that may complicate the interpretation of transgenic phenotypes, therefore careful controls and multiple transformation events are required, where possible, to separate transgene effects from somaclonal background variation. In contrast, tissue culture-derived cellular reprogramming may generate epigenetic states that are more permissive toward genetic transformation restrictions or genome editing, thereby increasing the efficiency of these biotechnological approaches and overcoming the limitations of recalcitrant species.

Somaclonal variation has led to some unexpected benefits in the conservation of endangered plant species (See how the article is brought back into consideration and the last snapshot is taken). Genetic fidelity, however, has been of paramount importance in conservation, leading to better protocols for ex situ conservation via tissue culture as a result of an understanding of somaclonal variation. In other contexts, somaclonal variation can be important to conservation through genetic diversity in small populations of endangered species, increasing possibility of adaptation and improved survival over the long term. This underscores the context-dependent value of somaclonal variation, which can be advantageous or disadvantageous depending on objectives of a particular conservation program. Emerging interest in plant-derived bioactive compounds and biomaterials has led to expansion of the pharmaceutical and industrial applications of somaclonal variation. For medicinal plants such as foxglove (Digitalis), Madagascar periwinkle (Catharanthus), and ginseng (Panax), variants with increased production of certain metabolites or new compound profiles have been produced. Specialized applications can be targeted through engineering of the fiber properties, oil composition, or starch characteristics of industrial crops. 21 However, the fact that somaclonal variation provides a powerful method for generating chemical diversity has not been fully appreciated particularly in the context of developing plant-based production systems for high-value compounds, which also include transgenic approaches or metabolic engineering. Somaclonal variation is primarily applied in breeding programs through a step-wise procedure. Tissue culturing to generate variation '! screening to select promising variants. Other plants are subject to field evaluation and agronomic performance, the stability of the desired characteristics and the absence of negative characteristics. Genetic bases of important traits were confirmed through molecular and cytogenetic studies and the overall genetic stability was determined. Finally, successful variants are integrated into breeding programs, or developed directly as new cultivars if are sufficiently superior and stable. This systematic method makes the most of somaclonal variation while controlling its native stochasticity. There are limitations and challenges facing somaclonal variation despite its numerous successful applications. Quantifying this process is complicated by the randomness specific to genetic mutations-following variation is usually random, with differences required to be screened in large populations to uncover valuable ones. The stability of detected phenotypic traits differs a great deal, with many reverting to the control or original

phenotype in later generations especially if induced by epigenetic changes rather than genetic changes

Unit 10: In-Vitro Production of Secondary Metabolites (Biotransformation)

Introduction to In-Vitro Production of Secondary Metabolites

Secondary metabolites are organic compounds that are not directly involved in the normal growth, development and reproduction of an organism. They are not essential for survival, unlike primary metabolites, but most often have important ecological function as defense compounds, attractants or signaling molecules. A wide variety of secondary metabolites are produced in nature by plants, fungi, bacteria and marine organisms, many of which have important pharmacological, agricultural and industrial uses. However, such approaches still face numerous challenges ranging from environmental variability to slow growth rates, low yields, and issues surrounding sustainability solely direct extraction from source organisms. Such restrictions have forced researchers to consider in-vitro methods for secondary metabolite synthesis. Controlled environments like in vitro systems allow modifications in cellular and enzymatic behaviours, favouring the in vivo production of compounds of interest. One important area of in-vitro production is biotransformation, which is the process of utilizing biological systems to convert substrate compounds into more useful products through enzymatic reactions. This methodology integrates the specificity and efficiency of biological catalysts with the controllable conditions of laboratory environments, offering several benefits compared to traditional extraction techniques. Over the last few decades, the field of in-vitro secondary metabolite production has developed considerably and has benefited from the advances made in plant tissue culture, microbial fermentation, enzyme technology and genetic engineering. These advances have broadened the scope of commodities and the efficiency of their synthesis. In vitro production has made obtaining many valuable secondary metabolites possible, including compounds from pharmaceutical agents such as paclitaxel and artemisinin, to food additives, fragrances, and industrial chemicals. The principles, methodologies, factors, and applications of in-vitro secondary metabolite production are discussed in this MODULE, especially biotransformation processes. A comprehensive understanding of such methods is also essential for researchers and biotechnologists

who want to tap into the immense potential of secondary metabolites in a sustainable and economically viable manner.

Biotransformation in Secondary Metabolite Production

Secondary metabolites are an extraordinarily diverse group of natural compounds that have evolved in organisms mainly as chemical adaptations to their environments. Secondary metabolites are typically not needed for growth and development like primary metabolites (amino acids, nucleic acids, sugars, and lipids) but instead are involved in ecological interactions defense against predators, pathogens, or competitors; attraction of pollinators or seed dispersers; or mediation of symbiotic relationships. There are estimated to be over 200,000 plant secondary metabolites (Nakanishi et al., 2005), and new compounds are still being discovered.

Background: Secondary metabolites have crucial importance pharmaceutical applications. Plant-derived compounds account for about 25% of prescribed drugs; these include paclitaxel (Taxol), derived from yew trees, for treating cancer; artemisinin, derived from sweet wormwood, for treating malaria; morphine, derived from poppies, for treating pain; and digoxin, derived from foxglove, for treating heart conditions. Aside from medicine, secondary metabolites are used as food additives, fragrances, pesticides, dyes and industrial raw materials. Their extraordinary diversity and utility have spurred considerable research efforts into their production approaches.

The classical method of extracting secondary metabolites has been mostly through direct extraction from original organisms. But this approach is fraught with challenges — they include:

- Impediments in the availability of source organisms in geographical or seasonal aspects
- The Complicated Story of Natural Sources
- Slower growth rates of many source organisms especially plants
- Variation in the production of metabolites across the environment

- Sustainability issues particularly for compounds that are sourced from endangered species

Complicated extraction and purification process

Crude extracts contain toxic or other unwanted component(s)

Such limitations have driven the exploration of in-vitro approaches to produce secondary metabolite. In vitro (Latin for "in glass") is the name of testing done outside the living organism, usually in the context of controlled laboratory environments and biological materials. Second, this covers approaches ranging from cultured cells and tissues to isolated enzymes and engineered microorganisms for secondary metabolite production.

In-vitro-production has a number of important benefits:

- Freedom from geographical, seasonal and environmental constraints
- Possibility of continuous production throughout the year
- Preservation of those endangered source species
- For fear of losing progress and to be able to optimize the conditions for high yield
- You are not very well trained on compounds that are hard to isolate from natural products

Easier extraction and purification processes

Lowered risk of contamination by toxic compounds

At least some promise of a scalable to industrial production

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 g 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

Methods for biotransformation and precursor feeding

Both have their own unique benefits and challenges, though the approach chosen will depend on the metabolite of interest, its biosynthetic pathway, the organism from which it is produced, and the scale required. More often, a mix of methods provides the best performing producing system. Production of secondary metabolites via in-vivo systems has continued to grow in importance and is now at an annual global market value of over $30 billion dollars exclusively for the pharmaceutical use of plant-derived secondary metabolites. With continuing technological advancement and cost reductions for in vitro methods, these are becoming increasingly preferred compared to conventional extraction methods, particularly for high value-on commodity compounds. Metabolic engineering, synthetic biology, and bioprocess optimization have great potential to improve the efficiency and applicability of these methods.

Microbial Taxa and their Role in Secondary Metabolite Production

Biotransformation is a model approach to the in-vitro production of secondary metabolites. It denotes the chemical modification of compounds by biological systems usually intact cells, tissues or purified enzymes. Unlike de novo biosynthesis in which organisms build complex molecules using simple building blocks or precursors, biotransformation refers to modification of existing substrate molecules. Proficient in catalyzing chemical reactions under mild conditions, this approach makes use of the unique abilities of biological systems to achieve highly specific, effective, and selective reaction modules that are often superior to classical chemistry for chemical biodiversity construction. Biotransformation itself has been utilized for thousands of years via fermentation processes for food and beverage production, but the term and its systematic application was only developed in the 20th century. The first documented biotransformation is often attributed to Louis Pasteur's discovery in 1858 that tartaric acid could be stereoselectively metabolised by microorganisms. Microbial transformations assumed industrial importance by around the mid 20th century with processes such as the 11á-hydroxylation of progesterone by species of Rhizopus, an important step in the synthesis of steroid drug compounds.

Biotransformation serves a few advantages, as listed below, in secondary metabolite production:

Stereoselectivity: While chemical reactions can yield different stereoisomers, biological systems are often able to determine and produce certain stereoisomeric forms of a compound, an important feature regarding many bioactive compounds, as their biological function can depend on stereochemistry.

Regioselectivity: Enzymes can direct modifications at a single position in complex molecules, enabling precise structural changes that would be impossible through chemical synthesis.

Mild reaction conditions: Biotransformations are often performed at room temperature, atmospheric pressure, and near-neutral pH which lowers energy requirements and preserves sensitive molecules otherwise decomposed by harsher reaction conditions.

One-step reactions: The complexity of many transformations that would require the execution of multiple chemical steps can often be achieved in a single enzymatic step.

Environmentally friendly: Biotransformations usually generate less toxic side products as compared to chemical syntheses and are possible in aqueous surroundings, which offsets environmental burden.

New derivatives of known compounds: Biotransformation is able to produce novel derivatives of compounds with the potential to exhibit a wider bioactivity profile or to have better pharmacological properties.

Biotransformation mechanisms involve a wide variety of chemical modifications, such as:

- Reactions of Oxidation (Hydroxylation, Epoxidation, Dehydrogenation)
- CARBON C-C bond forming/cleavingElimination reactions (dehydration, decarboxylation, elimination, Diels-Alder chemistry)RearrangementRearrangement reactions (isomerization)Oxidation-reductionC-H bond forming/cleaving oxidation reactions (C-H oxidation chemistry, C-O and C-C bond forming chemistry)Functional group reduction (alcohol, hydroxyl, aldehyde, alkyl)-Functional group oxidation (alcohol, hydroxyl, aldehyde, alkyne, alkene)Oxidation reduction reactions (carbonyl oxidation, double bond oxidation)
- Hydrolysis (ester and amide cleavage, glycoside cleavage)
- Conjugation (glycosylation, acylation, methylation, sulfation)
- Isomerisation (Structural rearrangement)

Reactions of condensation and addition

Dehalogenation, dealkylation deamination 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.

There are several important strategies for biotransformation in secondary metabolite production:

Microbial biotransformation: In this process, bacteria, yeasts, or filamentous fungi are used to transform substrate compounds. The benefits of microorganisms are rapid growth, simple cultivation, and genetic manipulation. Examples are the conversion of penicillin G to 6-aminopenicillanic acid by Escherichia coli expressing penicillin acylase (164), the transformation of terpenes by Pseudomonas species (43), and the modification of steroid structures by some fungi (162).

Biotransformation by plant cells: Plant cell cultures can carry out complex transformations, especially of plant-derived compounds. They often have unique enzymes not present in microorganisms, as well as be specific plant reaction catalysts. Cell cultures of Digitalis lanata can interconvert digitoxin to digoxin, and Catharanthus roseus cultures can hydroxylate vincristine.

Enzymatic biotransformation: Isolated or recombinant enzymes can be used for very specific transformations. This provides the highest control over reaction conditions and product purity, but such systems may need cofactor regeneration systems to obtain maximum efficiency. These include the production of vanillin from isolated dehydrogenases as well as lipase-catalyzed esterification reactions used for flavor compound synthesis.

Integrated biotransformation: More often than not, desired transformations are achieved by combining several biotransformation steps that involve a variety of biological systems. For example, hydrocortisone is produced through a series of varying enzymatic reactions of different microbial species.

Factors Influencing In-Vitro Production of Secondary Metabolites

The choice of a suitable biotransformation system is a complex one that depends on a number of factors:

- This chemical transformation that needs
- The substrate and product of interest can be very complex,

- Stability of substrate and product under biological conditions

Scale of production needed

Cost of substrates, growth media, and downstream processing are just a few of the economic aspects that need to be taken into account when considering economic aspects. Biotransformation has one primary application such as increasing the bioactivity or bioavailability of natural products. Many secondary metabolites in their native form show limited pharmaceutical utility owing to poor solubility, stability or bioavailability. This limitation can be overcome by biotransformation of both small and large molecules where selective modifications can be employed (e.g. glycosylation, hydroxylation, methylation). Fungal biotransformations leading to more potent paclitaxel derivatives have increased its anticancer efficacy, for example. Detoxification of plant secondary metabolites is also a significant application. Many toxins produced by plants act as defense mechanisms, and biotransformation can help to detoxify by generating non-toxic or less harmful derivatives. Cassava cyanogenic glycosides and different mycotoxins have been investigated and reported to follow this modulation. Biotransformation is also a useful tool in drug discovery and development. It enlarges the chemical space that can be screened and can provide lead compounds with better pharmacologic characteristics by producing novel derivatives. This approach is exemplified by the antibiotic erythromycin and its many semi-synthetic derivatives generated through biotransformation.

While biotransformation offers many advantages for secondary metabolite production, it faces a number of key challenges:

- Narrow substrate specificity of some biological systems
- 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
- Accelaration 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.

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, 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,

scalability, and suitability for bioreactor cultivation. Two prominent examples are the production of shikonin from Lithospermum erythrorhizon cell cultures at industrial scale in Japan and the production of berberine from Coptis japonica cell cultures. For metabolites that are produced only at higher concentrations in specialized cell types rather than in complex tissues or organs, cell suspensions are especially useful.

Hairy Root cultures (HRCs): These are type of root cultures which are developed by transforming plant tissue with the bacteria Agrobacterium rhizogenes (with the help of root inducing (Ri) plasmids). The T-DNA transferred by the bacteria integrates into the plant genome, leading to the overproduction of genetically modified roots featuring abnormal morphology and growth phenotypes. Through sustained high growth in hormone-absent media, hairy roots attain genetic and biochemical stability and, frequently, maintain or surpass the ability of natural roots to produce secondary metabolites. They have been used successfully to produce several root-derived compounds such as ginsenosides from Panax ginseng, tropane alkaloids from Atropa belladonna and artemisinin precursors from Artemisia annua. The shoot cultures are a productive system for producing secondary metabolites that naturally accumulate in the aboveground parts of the plants. An advantage of these organized cultures is that they are better differentiated than callus or cell suspensions and have a metabolite profile which is often more similar to that of intact plants. Other examples are digitoxin formation by Digitalis lanata shoot cultures, and artemisinin formation by Artemisia annua shoot cultures. Serial propagation of shoot tips or nodal segments on suitable media can be used to maintain shoot cultures.

Organ cultures: Not just roots and shoots, other organ cultures (like embryo cultures or flower cultures) can also be used for their specific organ metabolites. Vanilla flavour compounds, for example, have been produced in vanilla flower cultures, and certain cardiac glycosides accumulate in Digitalis embryo cultures.

Two-Phase Cultures: These systems utilize two separate culture phases; an initial growth phase for biomass accumulation, followed by a production phase in which environmental conditions are modified to promote metabolite synthesis. Single media change, growth regulators, temperature, or light conditions usually accompany the transition between different phases. This strategy has improved the production of

multiple metabolites in grape cell cultures (anthocyanins) and Coleus blumei cultures (rosmarinic acid) in a great proportion.

Integrated Microbial Systems for Fermentation

Some benefits of using microorganisms for secondary metabolite biosynthesis include rapid growth and relatively simple nutritional needs which allows a flexible adaptation to high scale fermentation systems. There are many flavors of that approach:

As part of the bacterial fermentation: countless secondary metabolites, especially from actinomycetes, such as the Streptomyces species, produce a wealth of valuable products such as antibiotics, immunosuppressants, anticancer agents, and enzyme inhibitors. Bacterial systems provide advantages in terms of fast growth, genetic amendability and well-established large-scale fermentation technologies. (3)Examples: Saccharopolyspora erythraea (erythromycin) and Streptomyces species (tetracyclines)

Fungal Metabolism: Filamentous fungi synthesize a wide range of secondary metabolites such as antibiotics (e.g. penicillins, cephalosporins), statins (e.g. lovastatin), immunosuppressive agents (e.g. cyclosporine), and mycotoxins. Most fungal fermentations are carried out using submerged culture in agitated bioreactors or, to a lesser extent, solid-state fermentation on agricultural residues or synthetic media. The successful industrial fermentations are penicillin from Penicillium chrysogenum and lovastatin from Aspergillus terreus.

Yeast Fermentation: Yeasts have the advantage of not only unicellular growth but also eukaryotic cellular organization; therefore these segregated and single-celled microorganisms are valuable hosts for post-genetic engineering plant-derived metabolites. Saccharomyces cerevisiae and Pichia pastoris have been genetically engineered to produce a number of plant secondary metabolites such as artemisinic acid (an artemisinin precursor), resveratrol and other flavonoids.

Cultivation of Endophytic Microorganisms: Since endophytes—microorganisms that reside inside plant tissues without causing noticeable disease—typically produce the same or similar secondary metabolites to that of host plants, The cultivation of such organisms may provide another source to replace plant extraction. Taxomyces

andreanae is an endophytic fungus that yields paclitaxel, and multiple endophytic fungi derived from Catharanthus roseus produce compounds similar to vincristine.

Enzyme-Based Systems

Second, isolated enzyme systems are the most controlled systems for cultural production of secondary metabolites, in terms of manipulation of reaction conditions independent of the cellular context:

Cell-Free Enzyme Systems: These utilize purified enzymes or cell extracts containing the requisite enzymes used to catalyze a target reaction or biosynthetic pathway Therefore, by abandoning cellular barriers and regulatory controls, they can maximize the reaction rates and yields of some transformations. For example, applications of isolated dehydrogenases for producing vanillin and glycosyltransferases for synthesizing diverse glycosides.

Subcategories of enzyme immobilization: Immobilized enzyme systems. This method has been exploited in diverse transformations to produce secondary metabolites, such as stereoselective reductions of carbonyls and regioselective glycosylations.

Cofactor regeneration systems: Most enzymatic transformations require usage of cofactors like NADPH, ATP or S-adenosylmethionine. These cofactors can be regenerated by systems that continuously replenish the cofactors in a reaction, increasing both reaction efficiency and economic viability. One example includes the use of coupling glucose dehydrogenase with NADPH-dependent reductases to continuously regenerate this expensive cofactor in biotransformations.

Low-Cost Genetic Engineering Methods

Novel molecular biology methods have facilitated the engineering of biosynthetic pathways to increase the production of secondary metabolites:

Therefore, metabolic engineering can be defined as genetically modifying the enzymatic, transport and regulatory functions in the cell so that it "produces more" desired metabolite. Such strategies can consist of overexpressing rate-limiting enzymes, deleting competing pathways, reducing feedback inhibition, increasing precursor supply, and

introducing efflux systems to alleviate product toxicity. Some key examples include refined engineering of yeast for artemisinic acid production and improved shikonin production in engineered L. erythrorhizon cell cultures.

Synthetic Biology: this is engineering applied to biology, designing and building new biological systems that work in predictable ways. In secondary metabolite production, it frequently entails the assembly of entire biosynthetic pathways in heterologous hosts. The reconstruction of the entire taxol biosynthetic route in yeast and the production of plant flavonoids in E. coli by co-expression of multiple plant genes are just a few examples.

Genomic analysis: tools such as CRISPR-Cas9 allow precise alterations of genomes, enabling biosynthetic pathways to be manipulated with unmatched precision. Such rational approaches have been utilized to optimize the production of diverse secondary metabolites, including artemisinin from Artemisia annua and tropane alkaloids from Atropa belladonna.

Mixing and Specialized Approaches

Many successful production systems integrate multiple approaches or use specialized techniques:

Culture Combinations: Co-cultures of different organisms can facilitate biotransformations that neither organism could complete on its own, or increase production via symbiotic interactions. Examples are co-cultures between plant cells and fungi or bacteria for production of complex alkaloids and the generation of complex transformation products using bacterial consortia.

Precursor supplementation: Supplementing cultures with biosynthetic precursors or intermediates to avoid rate-limiting steps and drive product formation. Mevalonic acid, for example, has been reported to enhance taxol production in Taxus cultures, whereas tryptamine enhances indole alkaloid synthesis in Catharanthus cultures.

Elicitation: Elicitors—compounds that trigger the plant defense response—can significantly increase the production of secondary metabolites in plant cell cultures.

These consist of biotic elicitors (e.g., fungal cell wall fragments, bacterial lipopolysaccharides) and abiotic elicitors (e.g., heavy metals, UV radiation, osmotic stress). For instance, when methyl jasmonate is applied, up to 50-fold increases in the production of paclitaxel in cultures of Taxus are obtained.

Permeabilization: Temporary disruption of cells membrane (permeabilization) can stimulate the release of the stationary phase accumulated secondary metabolites to the culture medium, allowing continuous production and simplified downstream processing. To achieve this, agents based on dimethyl sulfoxide, solvents and electric pulses have been used.

Immobilized Cell Systems: Immobilization of cells in polymer matrices (alginate, agarose, and polyacrylamide) or on solid supports often improves secondary metabolite production while also allowing the retention of cells in bioreactors. they have been successfully applied to Catharanthus roseus cells for alkaloid production, Penicillium cells for the production of penicillins and various plant and microbial cultures.

The choice of an appropriate method for in-vitro secondary metabolite production is influenced by many factors, including the chemical nature of the desired compound, its location in the metabolic pathways, the availability of precursor substrates, the complexity of enzymatic transformations required, and consequently the economic considerations. In such cases, a combination of different methodologies is often the most successful approach, customized to the exact needs of the target metabolite and production scale.

Key Factors Affecting Secondary Metabolites Production In-vitro

In-vitro production of secondary metabolites requires an exhaustive manipulation and optimizing of many factors to induce cellular metabolism and biosynthetic pathways. These parameters include biological, physical, chemical, and process-related factors that all could impact the yield and quality of the metabolites.

Biological Factors

Selection of Genotype and Source Material: The genotype of the source organism is fundamentally defined by its genetic content, which is reflected in its metabolic

capacities. Secondary metabolite production can vary greatly from species to variety and even individual plants or microbial strains. For example, the paclitaxel content can vary 10-fold among different Taxus species, and drug alkaloid profiles differ widely among Catharanthus roseus cultivars. The screening and selection of high-producing genotypes or strains of these biological systems are a crucial first step in developing efficient production systems (Wei et al. 2009). This is undoubtedly the case for microbial systems where mutagenesis and selection of high-producing strains traditionally led to large production increases, such as the 1000-fold increase in penicillin production in industrial Penicillium strains relative to wild isolates.

Cellular differentiation and development: The level of cellular differentiation has a very high impact on the secondary metabolite in plant systems. Berberine, morphine, and taxol—this same pattern repeats many times, with metabolites accumulating in specialized cells or tissues, whether that's stone cells for berberine, laticifers for morphine, or bark parenchyma cells for taxol. In vitro cultures typically have a lower production because of the absence of these specialized structures. That relationship between differentiation and production is what implicates the reason why organized cultures (hairy roots, shoots) often surpass undifferentiated cultures (callus, cell suspensions) for many metabolites. Grasping this relationship informs decision-making around which culture systems to use for individual compounds.

Growth Phase and Cell Cycle: A production of the secondary metabolites is often correlating with the specific growth phases or cell cycle stage. In many microbial fermentations, production initiates around the phase transition between exponential and stationary phase, a time marked by nutrient limitation and diminished primary metabolism. This phenomenon that's referred to as "growth-dissociated production" is the hallmark of synthesis of many antibiotics and other microbial metabolites. In plant cell cultures, metabolite accumulation also typically reaches a maximum when growth rates slow. Cell cycle analysis has demonstrated that the accumulation of some metabolites occurs mainly during certain cell cycle phases—anthocyanins in the G1 phase, alkaloids in the S phasewhich should aid in determining how to synchronize in order to accelerate production.

Cultural Age: As cultures age, their metabolic characteristics tend to alter. In plant cell cultures, habituation is a phenomenon that manifest as declining secondary metabolite production upon long-term subculturing. This happens as a result of epigenetic changes, somaclonal variation or selection for earlier-growing cells with lower secondary metabolism. However, certain types of fungi show improved production after long-term propagation with developmental status changes. Maintaining production requires careful monitoring and selection of suitable culture ages.

Microbial Contamination: Though mainly considered an issue in plant cell cultures, this can have a major effect on the output of secondary metabolites. Aside from the well-known negative impacts of both competition for nutrients and loss of culture, even subclinical contamination can change some metabolite profiles via evoked defense responses or through direct biotransformation of produced compounds. In longer-term cultures, it is imperative to adopt stringent aseptic techniques both in the laboratory and during media exchanges, and periodically reassess for contamination.

Physical Factors

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

Gaseous Environment (O, , CO,): 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, 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.

Agitation and Shear Stress Agitation helps improve the distribution of nutrients and gas exchange in liquid cultures, but also produces shear stresses that may harm the cells grown, notably plant cells with hard cell walls. For sensitive cultures, high shear stress not only hampers viability but also reduces productivity, whereas low shear translates into nutrient gradients, oxygen starvations, etc. In plant cell cultures, agitation speeds 60–100 rpm in flasks or moderate impeller speeds in bioreactors cause no net product yield reduction, while filamentous fungi form complex morphologies (pellets or mycelia) dependent on the conditions of agitation. Agitation needs to be optimized differently for each culture system, balancing shear damage and mass transfer benefits.

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, 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. Abscisic 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.

Elicitors and Precursor(s): Elicitors small molecule compounds inducing defence responses in plants showed an extraordinary potential in producing secondary

metabolites, especially in various plant systems. Examples for biotic elicitors are the fragments of the fungal cell wall (chitosan, glucans), bacterial compounds (lipopolysaccharides) and plant-derived molecules (oligogalacturonides). Such abiotic elicitors includes metal ions, UV radiation, and osmotic agents. Jasmonates and salicylic acid act as signaling elicitors, analogous to natural defense signals. Precursor feeding circumvents rate-limiting steps through supplying precursors that are closer to the final products (phenylalanine for phenylpropanoids, cholesterol for steroids, mevalonate for terpenes). Nonetheless, there are a few exceptions, combined elicitation and precursor feeding often yields synergistic productions improvement.

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 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.

Process-Related Factors

Culture System and Bioreactor Design It is well known that the physical setup of the culture system strongly impacts metabolite generation. Flask cultures are simple, but deliver little control whereas diverse bioreactor designs have been developed to increase control of those parameters. Stirred-tank reactors allow for homogenous conditions, but generate shear, airlift bioreactors give softer mixing and membrane bioreactors allow for perfusion and retension of cells. Bioreactor types such as wave-mixed, rotating drum and mist bioreactors are novel designs that are solving key challenges in alternative culture systems. Specialized bioreactors that allow three-dimensional growth are utilized under hairy roots and other organized cultures, leading to improved production efficiency. Immobilization Systems Cell or enzyme immobilization on solid supports or in polymer matrices has been reported to promote secondary metabolite synthesis. Immobilization decreases shear exposure, establishes microenvironments that favor the production,

product recovery, and maintenance of cells at high densities. Commonly used immobilization matrices include alginate, agarose, polyacrylamide, and diverse porous materials. These systems have been beneficial to plant cultures (increased alkaloid production from immobilized Catharanthus cells) and microbially, with increased antibiotic production (from immobilized Streptomyces). Product accumulation often negatively affects production via feedback inhibition or toxic effects on the producing organism. Such limitations can be relaxed by the use of two-phase systems with organic solvents, resins, or adsorbents that remove products selectively, as they are produced. A case in point is the use of amberlite resins to improve the production of taxol from the Taxus cultures by diafiltration to remove the product in an effort to increase yields, while organic solvent overlays are used to improve antibiotic production from bacterial fermentations. These strategies enable downstream processing while still providing some concentration of the product therein. Scaling Considerations Major challenges arise as operations scale from laboratory to industrial scale. Increased scale may also demand changes to optimized parameters owing to differences in mixing, gas transfer, temperature gradients, and shear profiles. Scale-up strategies involve keeping constant power input per volume, constant impeller tip speed or constant oxygen transfer coefficients. Each strategy has its merits, however, catering to different parameters and culture types—constant tip speed is beneficial to shear-sensitive plant cultures, while constant oxygen transfer helps aerobic microorganisms with high respiratory demands. Operational Mode (Batch, Fed-Batch, and Continuous) The production dynamics are also heavily influenced by the temporal modes of operations. Batch cultures are simple but have poor control on nutrient availability. Fed-batch operation, during which nutrients are administered in a controlled manner throughout cultivation, alleviates the limitation or inhibition by substrates and enables conditions for secondary metabolism to be achieved—this has improved production of substances in a wide range of systems such as paclitaxel in Taxus cultures and antibiotics in bacterial fermentations.

MCQs:

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

a) Increase genetic diversity

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

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

d) Germination

6. 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

7. What is an important factor affecting the production of secondary metabolites in-vitro?

a) Hormone concentration

b) Temperature

c) Light conditions

d) All of the above

8. Which of the following is a method of in-vitro production of secondary metabolites?

a) Callus culture

b) Somatic embryogenesis

c) Organogenesis

d) All of the above

9. What is the major application of haploid production in agriculture?

a) Seedless fruit production

b) Rapid genetic improvement

c) Increased photosynthesis

d) Enhanced drought resistance

10. In which of the following scenarios is somaclonal variation most likely to occur?

a) Tissue culture propagation

b) Natural plant breeding

c) Pollination

d) Controlled crossbreeding

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?
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.

Long Questions:

1. Explain the production of haploid cells using ovary and anther culture techniques.
2. Discuss the mechanisms and applications of somaclonal variation in plant breeding.

3. Describe the process and applications of in-vitro production of secondary metabolites.
4. Explain how somaclonal variation can be both beneficial and detrimental in plant breeding.
5. What are the challenges associated with haploid production in tissue culture?
6. Discuss the role of biotransformation in enhancing the production of secondary metabolites.
7. How can haploid plants be used to create homozygous lines for breeding?
8. Explain the factors influencing somaclonal variation in tissue culture.
9. Discuss the significance of secondary metabolites in pharmaceutical applications.
10. Describe the applications of haploids in accelerating crop improvement programs.

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 11 Protoplast Culture – Isolation, Regeneration, and Viability Test

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 interfect other wise). 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 cellwall, 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. The use of protoplasts can also be applied to somatic hybridization through cell fusion of cells from two different species, which presents a new variety of genetic combinations that can be formed above what would occur through conventional breeding. Utilizing protoplasts in genetic transformation helps biotechnologists to introduce desired genes to the plant and generate plants that possess attributes like disease resistance, stress tolerance, or nutritionally desirable traits. Protoplast culture has also played a key role in enhancing our knowledge about the regeneration of cell walls, cellular differentiation and the developmental processes in plants. Although these possibilities are amazing, protoplast culturing is often technically difficult (Hafez and Tatum, 1982). As protoplasts are naturally fragile, they must be handled with extreme care, and their surroundings must be engineered conditions to allow for short-term survival and regeneration. Isolation, maintenance of viability, division of protoplasts and regeneration of whole plants require an in-depth knowledge of plant physiology and optimization of protocols. Moreover, regeneration capacities for protoplasts vary greatly among species, with some plants easily generating new individual plants from protoplasts, while others remain recalcitrant despite significant experimentation. Herein, we highlight key challenges that have catalyzed ongoing advancements in protoplast technology, which in turn have led to well established protocols to broaden the scope of species that can be manipulated in this way. In the subsequent sections, we will discuss various aspects of protoplast culture, including the methodological details of protoplast isolation, factors that affect protoplast isolation, methods to assess protoplast viability, and to regenerate plants from this wall-less cells. These processes are of practical significance with biotechnological implications but also give an insight into basic cell biological and developmental traits of plants. The passage from an intact plant cell to a naked protoplast and back to a complete organism is one of the most spectacular examples of cellular plasticity which demonstrates the incredible potential present in each plant cell.

Isolation of Protoplasts

The isolation of protoplasts is an indispensable first step in protoplast culture technology; it demands a finely-tuned combination of 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. This approach uses a tailor-made cocktail of cell wall-degrading enzymes to decouple the various interconnected plant polysaccharide networks of the plant cell wall but preserves the plasma membrane . The most widely used enzymes are cellulase (to digest cellulose), hemicellulase (for hemicelluloses), pectinase or macerozyme (for pectin), and sometimes additional enzymes such as driselase or xylanase for particular components of the cell wall. Different types of plant material can be used to isolate protoplast, and which source material used will depend on the plant species being investigated and the research aims. One of the most frequently utilized tissues is leaf mesophyll cells, which is in part due to the easy isolation of this tissue and high yield of viable protoplasts (Li et al. 2021; Iwakuma Nature Biotech 2019). The protocol game typically involves a first step where young and healthy leaves are surfaced sterilized, next where the lower epidermis is peeled off exposing mesophyll cells to directly contact the enzyme solution. Other sources are cotyledons, hypocotyls, roots, calli, cell suspension cultures, reproductive tissues (e.g. pollen, embryos). The following tissue types, however, possess specific traits that affect the isolation methods and require the adjustment of factors such as concentration of enzymes, incubation times and form of purification. Enzymatic protoplast isolation is based on a standard protocol consisting of a series of sequential steps aimed are maximizing yield and minimizing cellular stress. The chosen plant material is first pretreated by herbal sterilisation, osmotic solution plasmolysis or mechanical slicing for better enzyme penetration. Then the tissue is placed in an enzyme solution with an appropriate composition of cell wall-degrading enzymes the composition varies with plant material. The osmotic potential of this

solution needs to be finely tuned deposited with mannitol/sorbitol/glucose — to ensure that the protoplast is not ruptured following cell wall release. Temperature (25-30°C), pH (5.5-6.0), and gentle agitation are controlled during incubation to enhance enzymatic activity and reduce cell damage. After proper incubation, between 3 to 24 hours depending on the type of tissue and enzyme combination, protoplasts released are in a solution with remaining undigested fragments of tissue, enzyme residues, and cellular debris. Purifying protoplasts from such a complex mixture represents a key step in the fractionation chain. Filtration through appropriate pore-sized meshes, centrifugation over density gradients (Ficoll, Percoll, sucrose), and isotonic wash passages are common purification methods. These protocols take advantage of differences in size, density, and sedimentation behavior to purify intact protoplasts from contaminants. These purified protoplasts can then be resuspended in appropriate culture media and concentrated to optimal amounts for downstream processes. Maintaining protoplast viability during the isolation stage is probably the biggest challenge. Protoplasts are exceedingly vulnerable to mechanical stress, osmotic shock, toxic molecules, and harsh environmental conditions. Thus, all solutions, devices and handling methods have to be optimized in order to reduce stress factors. In particular, the osmotic environment needs to be tightly controlled, because even small variations can cause protoplast lysis or plasmolysis. Isolation buffers can also be supplemented with antioxidants such as ascorbic acid, reducing agents to ameliorate oxidation-induced damage, etc. The timing of various steps is critical as well — long periods with enzymes or excessive handling during purification can take thousands of protoplasts viability down dramatically. The successful protoplast isolation hence relies on striking a delicate balance between adequate cell wall digestions and maintaining cell integrity. That balance differs widely among plant species, tissues, and developmental stages as well as growing conditions, so it is something that each specific application must hand-tune. The characteristics of isolated protoplasts, such as yield, viability, purity, and regenerative capacity, strongly affect all the subsequent tasks in protoplast culture and application. As a result, isolation of protoplasts is not only a prerequisite but also a key step that influences the success of protoplast-based research and biotechnological applications.

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 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, 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. 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.

Enzyme solution composition includes not only digestive enzymes, but also osmotic regulators, buffering agents, stabilizing compound. The osmotic environment played a crucial role in limiting protoplast release and survival during digestion. Main osmoticum in typical experiments is mannitol or sorbitol at a concentration of 0.4–0.8 M, depending on the plant species and tissue type. The osmotic pressure has to be determined experimentally for each system; too low values cause bursting of protoplasts, too high osmotic pressure causes plasmolysis and reduced activity of the enzymes. The pH of the enzyme solution, typically between 5.5 and 6.0, affects enzymatic activity and protoplast stability. Other ingredients commonly found in enzyme preparations are calcium ions to help condense membranes, antioxidants to minimize free radical injury and polyvinylpyrrolidone to absorb potentially poisonous phenolic things formed during digestive processes. It is stress conditions of enzymatic treatment determine the isolation results significantly. Temperature is another determinant of enzyme activity, with most protocols utilizing temperatures between 25°C and 30°C to maximize digestion efficiency while maintaining protoplast viability. Light conditions during isolation are also worth discussing—dark or dim light yields better results than bright light in raw membranes; light promotes the generation of oxygen radicals through photosynthesis that can attack exposed membranes. Different methods of agitations affect the rate of protoplasts release and the integrity after they were released. Ball or gentle orbital

shaking is normally advantageous by improving enzyme–substrate contact, whereas stronger agitation can cause mechanical damage to the released protoplasts. For some especially gentle, static incubation with occasional simple inversion might be the best method. After digestion, purification protocols to isolate protoplasts from remaining undigested tissue and cellular debris have a profound impact on both yield and quality. Whether via filtration through mesh or nets, or through the use of sieves larger fragments are rejected, but inappropriate implementation may damage sensitive cell types through shear. Centrifugation conditions, such as speed, duration, and the composition of density gradients, must be carefully optimized in order to achieve a clean separation while not exposing fragile protoplasts to g-forces beyond their tolerance limits. During the washing steps to remove the enzyme, the osmotic conditions must be performed so that lysis does not occur. Last but not least, along the way the final suspension medium for purified protoplasts, which is specifically prepared for maintaining these cells before they set into culture, should offer sufficient osmotic support and pH buffering as well as necessary nutrients to keep the protoplasts alive when they leap from a protoplast suspension towards a protoplast culture system. Given the complex interactions of these multiple factors, protoplast isolation protocols must be developed systematically. Researchers usually start from established protocols for related species or tissues and then systematically vary single parameters while assessing outcomes using yield measurements and viability assays. While sometimes tedious, this stepwise optimization process is still a critical component in generating high-quality protoplasts necessary for successful culture, regeneration, and experimental use. The major problems faced in protoplast isolation are due to the fundamental paradox of the procedure; breaking the protective cell wall whilst keeping intact the vulnerable plasma membrane along with cellular function. Successfully navigating this paradox in order to deliver high quality plant cells requires not only technical competence but also understanding of the biological factors that contribute to plant cell wall architecture and cellular responses to the stress of isolation procedures.

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 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. Other options for quantifying respiratory activity, and thus mitochondrial function in isolated protoplasts, are respirometric measurements based on oxygen consumption by means of oxygen electrodes or optical oxygen sensors. Analysis of chlorophyll fluorescence is a method for assessing the activity of photosystem II and the presence of functional chloroplasts in photosynthetic tissues, thereby indicating cellular health. Measurement of membrane potential takes advantage of the unique electrochemical gradient established and maintained across the plasma membrane of living cells. Dyes like DiBAC4 (3) or JC-1 are fluorescent potentiometric dyes that undergo either an alteration of distribution across the membrane or their spectral characteristics, thus enabling fast detection of this important physiological parameter132, 137. Intact protoplasts have a negative membrane potential between -120 and -200 mV, which dissipates following membrane damage or metabolic inhibition. The combination of flow cytometry with suitable fluorescent markers allows thousands of individual protoplasts to be quantified simultaneously for several viability parameters, yielding statistically significant information on the heterogeneity of the population and the attributes of different subpopulations.

Direct protoplast viability tests, though providing foundational knowledge, may not fully represent the true nature of protoplast quality, as successful division and regeneration are the eventual outcomes of interest. Plating efficiency, the percentage of isolated protoplasts that divide continuously to generate microcalli, provides a retrospective but clear viability determination. This is a parameter that subsumes all aspects of protoplast functionality necessary for successful culture and regeneration such as membrane integrity, metabolic activity and genetic competence. Protoplasts are generally cultivated in appropriate media under fine-tuned conditions and division

is monitored microscopically over a few days to weeks. The regeneration potential of primary cultured plant cells is often gauged with the first division of protoplasts cultured on enzyme-free (early) solid growth medium (2–7 d, depending on species). Collecting developmental data on the protoplast population from microcolony formation to plant regeneration is a more reliable and comprehensive approach. There are several key caveats that need to be taken into account when interpreting viability test results. Since different methods of viability assessment have different biological bases they may not give the same result on the same protoplast preparation. For example, the integrity of the membrane, although assessed in common tests of functional viability, can lead to overestimates of functional capacity, as it does not reflect metabolic competence or the ability to divide. On the other side of the redness index, highly stringent viability assays may also bias viability assessments low by only capturing nonviable states while not considering responses to acute environmental stressors that could recover during culture adaption. In addition, protoplast populations often show heterogeneity in both viability and regenerative potential, where subpopulations may have different characteristics despite being derived from what appeared to be homogeneous tissues. Therefore, the most comprehensive evaluation of protoplast quality is achieved by combining several viability assessment approaches. Practical implementation of viability testing in protoplast isolation and culture workflows relies on timing and choosing suitable methods. FDA staining, morphological examination and rapid assessment of isolation success is performed throughout the immediate process following the isolation. The metabolic tests may be performed at this stage or after an initial recovery period in order to be compensated for temporary isolation stress. Efficiency in such plating measures extends into the early culture period and therefore, despite being retrospective, provides definitive viability assessment. Standardized protocols of viability testing allow comparison between experiments, between researchers and between laboratories, which supports reproducible and robust methodologies in protoplast technology (for the research applications). Viability testing is not only critical for the assessment of quality, but also contributes to knowledge of basic research on cellular stress response, membrane biology, and factors affecting protoplast survival and regeneration. By associating viability parameters with isolation conditions, researchers can determine key stressors and design better protocols that

protect cellular integrity and function. In the same way monitoring viability as culture progresses shows what environmental and nutriitional dependencies must be fulfilled to maintain protoplast development. Such information is invaluable for practical applications in plant biotechnology but also for our general knowledge of plant cell physiology and the amazing rigidity and plasticity of plant cells in relation to protoplast culture systems.

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 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. During this wall regeneration period, osmotic conditions must be adequate—normally provided by culture media with mannitol, sorbitol or glucose, at concentrations that should avoid protoplast rupture but that permit osmodynamics adjustment on time as the wall develops. 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 factors secreted by surrounding cells promote progression through the cell cycle. Protoplasts can therefore exhibit both cell-to-cell signaling mechanisms dependent on the plating density and optimal densities between 104 and 106 protoplasts per milliliters depending on the species. During continued divisions microcolonies are formed and become visible as microcalli, the callus proliferation phase. In this step, the culture conditions will be gradually improved to release osmotic pressure and concentration of growth regulator which is a conducive factor of subsequent growth and preparation for morphogenesis will be established. It is at this phase when the transfer of liquid to solid media takes place, offering mechanical stability to embryonic cell aggregates. This also includes demands on nutrients, which change, trending towards more nitrogen sources, vitamins and microelements for an enhanced metabolism and differentiation. Callus features such as color, firmness, friability, and growth rate are often indicative of regenerative potential in the early stages, and ability to form embryos or organs is often correlated with unique morphological features like nodularity or pigmentation. This stage in developmental biology is referred to as morphogenesis, which is when growth becomes organized. Plant regeneration from protoplast-derived callus is achieved by two major routes: organogenesis and somatic embryogenesis. Formation of shoots usually occurs before development of roots during organogenesis, necessitating sequential treatment with cytokinin-rich, and then auxin-rich media. To determine this, the authors also explored the hormonal requirements across the pipeline, which revealed an initial inductive phase leading to

overall shoot meristem induction supplementary to a second phase reliant on root-targeting hormones required for root initiation (Section 3.3.3). Greening of callus, development of meristematic centers as nodular protrusions, and eventu- ally the emergence of leaf primordia represent visual signs of organogenic response. In contrast, somatic embryogenesis initiates embryo-like structures as they autonomously develop into entire plantlets with coordinated shoot and root formation. This route generally needs an initial treatment with high auxin concentrations followed by reduction or removal of auxin to induce embryo development. Embryogenic callus is usually more compact and organized than non-embryogenic tissue and contains typical proembryonic masses that can often be identified microscopically.

The last phase of regeneration also consists of establishment of functional shoot-root systems and acclimatization to ex vitro conditions. In the case of organs generated plants, differentiated shoots may need to be transferred to specific rooting media supplemented with necessary types and levels of auxins for the root induction. Whole plantlets must be gradually hardened by transferring sequentially to low sucrose and growth regulator-containing media and by exposure to decreasing relative humidity. Such reprogramming allows progression from heterotrophic growth at elevated humidity to autotrophic development at ambient conditions. Light intensity, photoperiod and temperature are important in regulating regeneration success throughout development, but requirements differ across some life stages. Lower light intensities generally favour early developmental stages, while high intensities are supportive of photosynthetic competence during later ontogeny. The genotypic effect on regeneration ability is one of the major problems to be solved in protoplast technology. Even within a species, you often see highly divergent regeneration potential among different varieties or cultivars from forming plants readily to being utterly recalcitrant. This genetic aspect requires protocol refinement for some genotypes and often restricts protoplast applications in crops of economic importance. Moreover, somaclonal variation, which is defined as genetic or epigenetic changes that happen during the protoplast culture process, can result in regenerated plants with changed characteristics. his phenomenon, occasionally exploited for the generation of novel variation, can disqualify the use of regenerated material for applications in which genetic fidelity is a requirement, thus the need to screen and select regenerated material carefully. Several methods have been established

to optimize success at regeneration for such recalcitrant species or genotypes. Processing and/or chemical treatments using compounds like phloroglucinol, silver nitrate, or antioxidants can alleviate certain metabolic or developmental barriers. For some species, physical methods, such as adjustments of light quality, temperature cycling or electrical stimulation, have been slightly more promising. Genetic engineering strategies aimed at key developmental regulators or stress response pathways are promising longer-term solutions. Additionally, transcriptomic, proteomic, and metabolomic analysis to understand the molecular basis of regeneration competence provides important information for rational improvement of protocols. Isolated protoplasts can be re-generated into plants but it is a difficult process, however, it has great potential for improvement and research of plants. This is because somatic hybridization via protoplast fusion allows for the generation of new genetic combinations that cannot be made using traditional breeding. Protoplasts can be genetically transformed, enabling modification at high precision without influence from Agrobacterium host range limitation. Protoplast-derived colonies can be selected directly at the cellular level for desired characteristics such as disease resistance or stress tolerance. Moreover, the protoplast-to-plant regeneration system is a good model to study general aspects of plant development like cellular totipotency, differentiation, and morphogenesis. The ongoing improvement of regeneration systems and understanding of fundamental molecular mechanisms leads to an ever better species spectrum notified by this powerful technology not only for basic plant research but also for applied agricultural biotechnologies.

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. Microfluidic systems for protoplast handling allow for fine-tuned control of the microenvironment of single cells, which could address issues related to traditional bulk culture techniques. Machine learning pertaining to the fields of artificial intelligence and automated imaging and data analysis synergized holds the key in terms of predictive modeling of protoplast responses and protocol optimization with minimum empirical trials. As our understanding of the molecular basis of totipotency and cellular reprogramming improves, this approach could be used to unlock regeneration potential in currently recalcitrant species by manipulating key developmental regulators in a specific manner. Identifying specific transcription factors, epigenetic regulators, and signaling pathways regulating the differentiation from protoplasts to plants provides logical targets toward bridging the regeneration gap. Additionally, the development of synthetic biology strategies allowing the design of artificial regulatory circuits governing development has the potential to fundamentally change the manner in which regeneration of protoplasts is achieved. We have seen developed these applications not only also in the new research as: Protoplasts provide unique experimental systems for quantitative studies of cellular responses to beneficial or pathogenic microorganisms, bypassing the barrier of the cell wall (100). For example, in metabolic engineering, various protoplast-based screening approaches facilitate the high-throughput evaluation of genetic manipulations directed at biosynthetic pathways. In the conservation of biodiversity, the protoplast technologies offer alternative methods for conservation when the requirement for regeneration through

conventional methods proves difficult in rare and endangered plant species. We can foresee the evolution of protoplast culture technology in the coming years most likely paralleling trends in biotechnology as a whole, trending towards more precision, higher throughput, deeper understanding at the molecular level, and wider applications. In the course of these developments, protoplasts will continue to be key tools in our studies of plant cellular biology and our utilization of plant genetic diversity for sustainable crop production and environmental stewardship. The naked plant cell, devoid of its wall but preserved in its secrets of development, continues to provide remarkable insights into the nature of plant life, and extraordinary opportunities for humans for exploration and creativity in plant science and technology.

Unit 12: Somatic Hybridization

Introduction to Somatic Hybridization

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 pl… This approach was developed in the 1970s when scientists began exploring ways to circumvent the limitations of traditional sexual hybridization that is frequently restricted by reproductive incompatibility between distantly related species. The first successful somatic hybrid was produced in 1972 when Carlson and coworkers fused tobacco mesophyll protoplasts which proved that this concept can be useful in improving 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.

Somatic hybridization is based on several stages of isolation of viable protoplasts from donor plants, protoplast fusion, selection of hybrid cells, regeneration of hybrid plants, and verification of their genetic composition. There are challenges at each stage and the need to optimally tune the workflow at each stage to make this work. The fusion itself can also be done through a number of different approaches, using either polyethylene glycol (PEG) treatment, electrofusion, or micromanipulation, with each approach having its advantages and disadvantages for the given plant species. Somatic hybridization has progressed from a laboratory curiosity into a versatile tool for harnessing genetic diversity in plant breeding programs around the world over several decades. Innovations in protoplast isolation protocols, fusion procedures, and selection procedures have made it applicable to a greater variety of plant species. Additionally, combining somatic hybridization with other biotechnological techniques, including genetic transformation and marker-assisted selection, has significantly increased its accuracy and efficacy in introducing specific traits of interest. In spite its promise, somatic hybridization has some limitations that have prevented its use in commercial breeding programs. These advantages are counterbalanced by the disadvantages of the recalcitrance of some plant species to protoplast culture; the difficulties encountered in regenerating viable plants from hybrid cells; genetic instability in regenerated hybrids; and the unpredictable expression of the combined traits. Nuclear fusion also has the potential to generate undesirable gene combinations in a random manner, and in such cases, extensive backcrossing may be necessary to remove unwanted traits. Somatic hybridization requires efficient protoplast isolation from different plant species, but the efficiency can be limited due to lack of tools or methods for some plant species. The integration of these emerging technologies with somatic hybridization is_ likely to improve the resolution and effectiveness of the process, allowing for greater application in crop improvement goals, as these technologies

become more readily available and applicable. This holds true especially now that the world is grappling with global challenges in the face of population growth, climate change and reductions in agricultural resources — and somatic hybridization has been considered a key step toward the generation of robust and productive crops. This technique could play a crucial role in sustainable agriculture and food security in the coming decades, utilizing the genetic diversity present in wild relatives and landraces.

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 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 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).

Creating isolation medium involves not only various osmotic stabilizers, but also other inorganic salts, buffers, and protective agents. Calcium ions help stabilize the plasma membranes and reduce the aggregation of protoplasts, and magnesium ions function as cofactors for enzymes. Buffers such as MES (2-(N-morpholino) ethanesulfonic acid) or phosphate keep pH close to the optimal range where they are active (generally pH 5.5-6.0). For example, ascorbic acid, cysteine or dithiothreitol, are often added as antioxidants to neutralize harmful reactive oxygen species generated during isolation. Certain protocols add bovine serum albumin or polyvinylpyrrolidone to bind to environmental phenolic compounds released from broken cells to inhibit oxidation and toxicity related to phenolic compounds. Enzymatic digestion is inherently dependent on physical conditions such as temperature, time, agitation, and design of the vessel and these factors have a strong effect on both protoplast yield and quality. Typically, digestions are performed in the range of 25-30°C for 3-16 hours, depending on tissue type and enzymes to be used. Mild shaking aids enzymes by allowing them to reach cell walls while keeping mechanical damage to developing protoplasts at a minimum. Depending on the scale and specific

requirements of the isolation, various vessels are used from simple petri dishes to complex digestion chambers with controlled environments. Protoplasts need to be separated from undigested tissue, debris and spent enzyme solution after digestion. This purification normally includes multiple filtration and centrifugation steps. Next, low-speed (50–100 × g) centrifugation pellets protoplasts and allows smaller debris to remain in suspension. Protoplasts can be further purified and separated based on size or density valuable properties when isolating protoplast from mixed tissue sources by subsequent resuspension and centrifugation in fresh medium, sometimes with density gradient materials, such as sucrose, Ficoll, or Percoll. Before carrying out fusion, it is essential to assess the quality of the protoplast. Immediate feedback is provided by viability tests with vital stains like fluorescein diacetate that will result in the conversion of nonfluorescent fluorescein to fluorescent fluorescein by the action of esterases residing in living cells, allowing a determination of the percentage of viable protoplasts. The yield determination performed by hemocytometer counting enables to calculate the isolation efficiency and will guide the following of the used fusion protocols. Microscopic study showed the intactness of nuclei, chloroplasts and other organelles, confirming protoplast integrity. Certain protocols include other assays to assess metabolic activity, membrane integrity or division capacity, which provides a more comprehensive assessment of protoplast quality. Protoplast isolations are species specific and there are challenges. For example, monocots, which include cereals and grasses, are less likely to be hard to fracture than dicots, because of their silica-rich and more recalcitrant cell walls. Phenolics and other secondary metabolites in woody species inhibit enzyme activity and become cytotoxic when released. For highly recalcitrant materials, such as tough tissues, enzyme mixtures, antioxidant additions, and mechanical interventions have been suggested.

Developments Recent advances in protoplast isolation techniques Enzymes have been developed in highly purified, characterized preparations to be used instead of crude mixtures when feasible, leading to greater reproducibility and less toxicity. Technologies for sampling plant tissues, enzymatic digestion, and protoplast purification have increasingly been automated, leading to higher throughput, greater standardization and improved reporting of experimental conditions. Microfluidic devices offer precise manipulation of small samples and single protoplasts, which are especially useful for

rare or hard-to-isolate species. These advances together widen the list of plants accessible to protoplast-based engineering or interventions. Directly after isolation, protoplasts must be handled in a manner conducive to their fusion competence. Protoplasts freshly isolated tend to fuse better than protoplasts preserved in culture for long times. But some protocols include a 1-24 hour "recovery" period so that membranes can repair and stabilize before fusing. For the time being, protoplasts are usually kept on a nutrient-deficient, osmoticum medium of high osmolality at low temperature (4–18 °C) in order to suppress metabolical activity while stimulating the healing of membranes. Another factor by producing hybrid plants by the isolation of protoplast from two or more parental species for hybridization. Despite differing in optimal isolation protocols, the parental protoplasts must be separated under conditions that provide equivalent viability and fusion competence. At least some fusion techniques require distinguishable protoplast populations, usually by virtue of intrinsic characteristics (i.e., chloroplast presence/absence) or exogenously introduced markers such as fluorescent dyes. Simultaneous availability of fusion-ready protoplasts from various origins frequently require staggered commencement of isolation approaches, which are dependent upon their individual routine. In summary, protoplast isolation is an advanced mechanism that demands ratiocination of different variables for each plant species and tissue type. The potential for effective somatic hybridization is directly influenced by the quality of isolated protoplasts and will dictate the efficiency of subsequent fusion events. The evolution of isolation processes, coupled with a growing understanding of plant cell biology, and advances in technologies, have successively extended this protoplast-based genetic transformation to an increasing number of plant species, allowing somatic hybridization for crop improvement.

Mechanism of Somatic Hybridization

Somatic hybridization is followed by a cascade of molecular and cellular events that influence the success and nature of hybrids formed starting from protoplast fusion, leading to hybrid plant regeneration. Characterization and understanding of these mechanisms are key to optimizing protocols and predicting the genetic constitution of resultant hybrids, thus improving the power and utility of this technique in breeding programs. Various procedures that cause transient destabilization of the plasma

membranes lead to the fusion of adjacent protoplasts and protoplast fusion is the crucial step in somatic hybridization. Chemical fusion, especially polyethylene glycol (PEG), is the most commonly used method owing to its ease-of-application and applicability to a variety of plant species. PEG enhances membrane fusion via multiple proposed mechanisms: it induces dehydration at the protoplast surface, resulting in close proximity of the membranes; it alters membrane lipid organization, increasing fluidity; it removes the negatively charged domain that repels protoplasts; and it also has the potential to form molecular bridges between neighboring membranes. PEG-induced fusion efficiency is governed by multiple factors, including PEG molecular weight (1500-6000 Da), concentration (25-50%), exposure times, pH, temperature and divalent cations, like calcium, which promote membrane destabilization followed by membrane restructuring Electrofusion is another method that creates temporary pores in membranes via electropermeabilization using short electrical impulses. The principles consist of two steps: protoplasts are aligned into chains by diffuse electro-polarization between two electrodes in a parallel chamber via dielectrophoresis using an alternative current, and then subjected to electroporation with short repetitive high-voltage direct current pulses creating pores in the aligned membranes, thus inducing membrane fusion at contact points. Compared to chemical methods, electrofusion offers several advantages such as better control of fusion parameters, higher fusion frequencies, lower toxicity, and minimal disruption of protoplast contents. It does, however, demand specific apparatus and prudent optimization of electrical parameters (field strength, pulse length, and frequency) for every protoplast combine. Other methods for fusion that are not widely used are laser-mediated fusion, focused laser beams generate by membrane disruptions; microinjection is a mechanical fusion method using micropipettes can directly use micropipettes induce the fusion; and the other chemical agents gather sodium nitrate, dextran, liposome, etc. JUXTAPO, COPY-COUNT, or MNAP: Each method has its own advantages and limitations, and the choice of which to use will ultimately depend on specific plant species needs and research goals. An isolation step to examine heterokaryons the cells possessing nuclei from both parental protoplasts — must be performed after fusion treatment to separate these cells from a mixed population, typically composed of unfused parental protoplasts and homokaryons (self-fused protoplasts). To overcome this issue, multiple

selection strategies have been proposed . Morphological differences between parental protoplasts can be utilized for the visual selection based on their size, chloroplast content, or incorporated dyes (fluorescent or colorimetric). Flow cytometry allows for this visual parameter to be used for automated sorting, greatly increasing throughput and accuracy. Although micromanipulation provides direct physical isolation of identified heterokaryons, it is labor-intensive and not high-throughput. A more elegant selection strategy is via genetic complementation, where both parental lines carry a distinct selectable marker or auxotrophic mutation. Only heterokaryons contain the full complement of genes required for survival when selective conditions are met. For example, one parent may carry resistance to antibiotic A, while the other parent carries resistance to antibiotic B; only in medium containing both antibiotics will hybrid cells survive. In the same manner, metabolic complementation may use parents with a diverse metabolic need, whereby only hybrids would grow on minimal medium.

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 preferened amplification of one mais 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. —Whole _

cybrids with nuclear genome type predominantly determined by one parent and cytoplasmic (organellar) type determined by the other parent combining cytoplasmic manipulations with phenotypic transfer (eg, use for transferring male sterility and herbicide resistance). Changes in genome structure following protoplast fusion are not just a simple addition of parental chromosomes. Genome instability resulting in chromosome elimination, rearrangement or recombination is common therefore somatic hybrids. These changes may take place right after fusion or during later cell divisions and plant regeneration. Due to the removal of the chromosomes of one parent, especially in asymmetric hybrids, partial hybrids generally consist of most chromosomes from one parent with only several chromosomes from the other. Chromosome fragmentation and rearrangement create new configurations that were absent in either parent. Homoeologous recombination occurs between the chromosomes from the two different parents at somatic cells, and this process led to novel genetic combinations. Some changes, such as histone modification and changes to DNA methylation patterns, are epigenetics: changes to how gene expression is regulated without any alterations to the DNA itself. While these genomic changes lead to novel phenotypes in somatic hybrid populations, instability and non-uniformity in regenerated plants can also occur. The regeneration of hybrid plants from protoplasts that have been fused is maybe the most difficult part of somatic hybridization. It is initiated with sustained cell division to develop microcalli, and subsequently through morphogenesis or embryogenesis generates organized structures (shoots and roots). Each stage demands unique hormonal and nutritional conditions, which vary greatly between species. The totipotency of plant cells — i.e. their ability to redifferentiate into whole plants — provides the theoretical underpinning for regeneration. However, practical limitations exist, especially for monocots, woody species and some recalcitrant dicots. Protoplast source tissue, culture conditions, genetic factors, and stressing by protoplast isolation and fusion conditions all play a role in regeneration efficiency. Another cloud variable is that fused cells also have mixed genomes, which can lead to uneven amounts of gene expression or incompatible regulatory networks, making it more difficult for normal developmental processes to take place. Verification of hybridity in regenerated plants is a crucial step in somatic hybridization protocols. Morphological features offer a first strike but are often highly variable because of environmental conditions and the unpredictable expression of combined features. Chromosome counting and

karyotyping were performed based on cytological techniques and provided only information regarding the number and gross morphology of the chromosomes without resolution for genetic analysis. Isozyme analysis, which quantifies the electrophoretic mobility of enzymes with multiple forms, can be used to demonstrate that protein variants from both parents are present in a hybrid. DNA-based molecular markers (including RFLPs, AFLPs, SSRs, and SNPs) provide the most reliable types of markers. These methods are able to determine exactly what percentage of the nuclear genome each parent contributes. In case of organellar genomes, their parental origin or recombinant nature can be evaluated, based on specific markers or sequencing of diagnostic regions.

The underlying mechanisms of somatic incompatibility is an area of active research that has practical implications for hybridization success. Incompatibility between different species during prefusion could also be due to increased heterogeneity in protoplast size, altered membrane composition, or differences in surface properties that block membrane fusion. Intrinsically nonsynchronous karyogamy or anaphase lead to post-fusion nuclear incompatibility due to heterogeneity in chromosome number or structure or cell cycle timing. The regulatory incompatibility arises when mismatched gene expression networks produce contradictory signals, thus, leading to a disruption of cellular homeostasis or developmental programs. Incompatibility of nuclear-encoded proteins with their respective organellar genomes from another evolutionary lineage leads to malfunction of key processes such as photosynthesis or respiration. This knowledge leads to the development of strategies to address these incompatibilities that include the use of intermediary compatible bridge species, use of technologies that effect asymmetric fusion to minimize the contribution of foreign genetic material or treatments which minimize the impact of negative interactions. Somatic hybridization mechanisms are continually expanding with the advances in technology. Imaging technologies such as confocal microscopy and transmission electron microscopy reveal the morphology of both fusion events and downstream cellular reorganizations with high resolution. Flow cytometry and sorting excel at analysing and purifying fusion products based on multiple criteria. Genomic and transcriptomic (next-generation sequencing) profiling has also uncovered extensive genetic changes that have occurred post hybridization at the chromosome level (karyotype remodeling), gene expression

level, and via epigenetic changes. Overall, these technologies extend our knowledge of core processes that drive somatic hybridization, and also offer useful means of improving somatic hybridization efficiency and applicability. Mechanistic knowledge enables useful applications in many new ways. Through asymmetric somatic hybridization, interspecific genetic transfer can be achieved with reduced genetic load from the donor, by breaking up one of the parental genomes by irradiation or chemical treatment prior to fusion (Khan, 2023). Micronucleation-mediated chromosome transer enables the addition of individual chromosomes or chromosome segments. Targeted introgression introduces genes of interest by combining somatic hybridization with molecular selection in order to move specific genes between species. Organelle transfer methods aim to replace or modify mt or cp genomes, with limited modifications to the nucleus. Such applications harness insights on fusion mechanism to deliver more accurate genetic modifications towards real-world breeding goals. Overall, the somatic hybridization mechanism involves multiple cell and molecular events from membrane fusion to plant regeneration. Numerous factors, such as the particular method of fusion, the strategy used for selection, interactions between the nuclei and organelles, and genomic integrity versus regenerative capacity, influence this outcome. Further study of these mechanisms, together with ongoing improvements in cell manipulation and genetic analysis technology, increasingly makes somatic hybridization an effective tool for plant improvement programs.

Unit 13: Introduction to Fusion of Protoplasts

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 relicated 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 technique which has been developed on fundamental concepts established in the early 20th century, and evolved significantly betwee the 1960s and

1970s. The successful generation of the first somatic hybrid plants by scientists such as Peter Carlson showed that this technique was indeed likewise practical. These initial successes spurred a flurry of advancements in the field and soon after, protoplast fusion became an essential tool in plant biotechnology. Protoplast fusion has wide applications in agriculture and biotechnology. This approach allows for the cross-species transfer of favorably useful traits, including disease resistance, stress tolerance, and improved nutritional quality. 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. In this way, thousands of genes can be transferred in one go, including perhaps novel combinations of genes that could never be brought together using targeted gene transfer methods. Protoplast fusion also retains organelle genome integrity, allowing trait transfer through these organelle genomes (Chloroplasts and Mitochondria). Beyond its direct applications, the importance of protoplast fusion can not be overstated. This process allows for the viability of new genetic configurations to exist to improve the genetic arsenal available for crops capable of solving issues necessary for the future food supply, adaptation to changing climates, and viable agriculture practices. The true utility of any of these advantages would be reliant on a number of technical and biological hurdles being addressed including the establishment of efficient protocols for protoplast isolation, fusion and subsequently regeneration of viable hybrid plants.

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 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. These pores gradually widen, resulting in the fusion of the plasma membranes and the fusion of both protoplasts, forming a single, common membrane that encircles the contents of both protoplasts. Membrane fusion on a molecular scale involves the specialized interactions of membrane proteins and lipids. Calcium ions ($Ca^{2}z$) are important participants in this process, as they serve as a bridge connecting negatively charged phospholipid head groups and decreasing electrostatic repulsion to facilitate membrane adhesion. Calcium ions also trigger certain proteins that associate with the membrane and promote the rearrangement of membrane-bound proteins critical for guiding membrane components to where they will interact and complete the fusion process. Though the exact molecular mechanisms of these effects mediated by calcium are yet to be fully understood, they are thought to include changes in membrane fluidity and activation of calcium-sensitive enzymes. After membrane fusion, karyogamy leads to nuclear fusion in the heterokaryons (which are formed by the fusion of the nuclei from both parent cells). In this case, the nuclear envelope of the parental nuclei disintegrates during karyogamy, intermixing the chromosomes, and later reorganizing into one nucleus containing the genetic material of both parents. Karyogamy, which occurs during this nuclear fusion event, is crucial for the stable incorporation of the parental genomes, but in certain instances especially in asymmetric fusion when one genome is actively sought to be eradicated — may not be fully realized or is selectively inhibited. The last step in the

fusion process includes remodeling cytoplasmic materials, comprising organelles as well as cytoskeletal parts, into a practical hybrid cell. This reorganization implies that cellular compartments are not passively redistributed by diffusion but actively collapsed and removed, targeted by selective retention or elimination. In the case of some of the fusion protocols aimed at transferring particular organelle genomes (e.g. chloroplasts or mitochondria), conditions are optimized to promote the retention of these organelles from one parent at the cost of the other ones. This selective retention also is an active and complex process involving nuclear-genome-organelle-factor interactions. Not all fusion events give rise to "heavy" hybrid cells, however. The rate of success of fusion and the subsequent development of hybrid cells is influenced by a variety of factors, including the compatibility of the parental genomes, the physiological state of the protoplasts, and the conditions under which the fusion takes place. Insight into these factors and the inherent mechanisms of fusion is important to developing effective protocols specific to an application.

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, 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 membrane4 surface and pulls protoplasts together, resulting in DNA driven2 fusion by the destabilization3 of membrane lipid motifs at areas of protoplast contact. Usually, calcium ions greatly facilitate membrane adherence and fusion, enhancing the fusion process. Although PEG-mediated fusion is straightforward to conduct and economical, fusion efficiency can be variable and is potentially toxic to some protoplasts, requiring careful optimization of PEG concentration, molecular weight, exposure time, and post-

treatment conditions (e.g. Ko et al., 2020; Li et al., 2018). Other chemical agents employed for protoplast fusion beyond PEG include sodium nitrate, which modifies membrane permeability; dextran for protoplast aggregation; and several lipophilic compounds that directly affect membrane lipids. Although less common than PEG due to limitations in efficacy or specificity, these alternative chemical fusogens are often used in specialized applications where PEG is less effective or more cytotoxic. Protoplast fusion is a physical method that generally applies mechanical forces to closely associate protoplasts and promote membrane fusion through mechanical forces. Electrofusion is a particularly prominent approach among them. Electrofusion employs short, high-voltage pulses of electricity to electroporate (permanently open the plasma membrane) the cells for a small amount of time as well as use dielectrophoretic forces to get protoplasts to align and fuse together. Advantages of electrofusion are such as high efficiency, reproducibility, and precise control of fusion parameters. In addition, electrofusion tends to generate fewer multinucleate fusion products than chemical fusion methods, which may be desirable for specific applications. Unfortunately, the need for specialized equipment and the susceptibility to electrical damage of cellular components make this technique not widely used. Physical methods use less specific forces, inducing protoplast merger by direct contact or physical interaction, such as micromanipulation, microfluidic devices, and laser induced fusion. These more specialized physical modalities provide high precision and metrology but are by and large lower throughput and require complex apparatus. Somatic hybridization techniques that integrate fusion with selection strategies to detect and isolate hybrid cells have been a major development in protoplast fusion methodology. These methods are especially critical as fusion events tend to happen at relatively low frequencies, and the generated fusion products are closely associated with unfused protoplasts and homokaryon (self-fused) cells. Next, various selection approaches have been utilized for hybrid formation, including complementation systems in which parental cells harbor different genetic deficiencies that are complemented in hybrids, antibiotic resistance markers such that hybrids retain resistance genes from both parents, and visual markers that allow identification of hybrids through dual marker fluorescence, such as separately expressed fluorescent proteins in different parental lines.

Microfluidic platforms for high-throughput, controlled fusion of protoplasts, as well as integration of imaging technologies for real-time monitoring of fusion, have also

been recent innovations in protoplast fusion methodology, in addition to the application of computational modeling to achieve optimal fusion parameters. These advancements are further improving the accuracy, efficiency, and accessibility of protoplast fusion methods, enabling novel applications for crop modification and biotechnology applications. Depending on the species types, the goals of the fusion, availability of support and resources for the fusion, and the characteristics of the fusion products for downstream applications, different optimal fusion methods may be selected. Often the best approach will be a combination of these methods, or a sequential approach where different techniques are used at varying points in the fusion process.

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 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. The interaction between pegylated nanoparticles and membranes is greatly influenced by the molecular weight of PEG, with high molecular weight variants generally having a better ability to interact at the membrane than low molecular weight variants, although they might be more toxic 68. The extent of dehydration and membrane destabilization induced by PEG varies with the concentration of PEG, with optimal concentrations for destabilization on 30-50% depending on the species 13. The exposure time to PEG must provide enough time for fusion while minimizing toxicity which most commonly is done in 10-30 minutes. The pH of the fusion medium also modifies the action of PEG on the membranes: fusion is generally favored at alkaline (pH 7.5–8.5) conditions. Divalent cations, especially calcium, promote membrane attachment and fusion, but calcium at high enough concentrations can cause irreversible membrane lysis. For electrofusion techniques, important characteristics are the strength and duration of the voltage pulses, as well as their frequency. The field strength should be enough to promote the changes of membrane permeabilization without inducing irreparable damage and is normally in the range of 500–1,500 V/cm. The pulse number and pulse duration determine the extent of the membrane disruption, and in many instances, several short pulses provide a more effective treatment than a single long pulse. The ionic strength and conductivity of the electrofusion medium strongly influence electric field distribution and protoplast response to electrical stimulation. The conditions of the culture after the fusion play a crucial role in determining the survival, division, and regeneration of the fusion products. As protoplasts need to recover from their fusion stress and eventually divide, both the nutrients, hormones and osmoticum content of culture medium must support this process. Other aspects of the culture environment, including temperature, light conditions and pH, can influence the viability and development of fusion products. As fusion products grow and their walls regrow, the osmotic pressure in the culture medium should be gradually decreased to stabilize them. Exploring hybrid compatibility between fusion partners is a foundational determinant dictating hybrid viability and realization of development. When these constitute another independent evolutionary geneline,

such genomic divergence leads to the development of chromosomal instability, aberrant gene expression, and aberrant development of hybrids. Nuclear-cytoplasmic incompatibilities, in which one species' nuclear genome is incompatible with another species' organelle genomes, can therefore lead to dysfunction of important cellular processes. These compatibility issues can, in part, be solved with preferential asymmetric fusion techniques that enrich for the loss of a parental genome which permits the transfer of specific traits and significantly reduces the genetic incompatibility. The ratio of parental protoplasts, protoplast density at the time of fusion, and the timing of fusion with respect to protoplast isolation are methodological factors affecting fusion outcomes. Increasing the density of protoplasts typically raises the rate of fusion events, but can also facilitate unwanted multiple fusions. a consideration for the fraction of parental protoplasts to be fused will also depend on the specific goals for the fusion experiment, and also the relative fusibility level of each parent. This set of dynamic factors collectively develops a multifactorial parameter space for optimizing protoplast fusion depending on the types of applications employed. Empirical optimization via factorial experimental designs is usually required to discover the best conditions for a specific fusion system. The need for structured protocols and reporting formats noted previously can improve reproducibility and the ability to compare data across studies and laboratories.

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 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. Many of these wide hybrids are sterile, and/or fertility reduced, but means such as chromosome doubling (to amphidiploids) or back-crossing (to introgress particular traits into a certain parental background) can sometimes mitigate these issues, finding stable, fertile lines with commercial potential. Another target for transfer via protoplast fusion are cytoplasmic traits, which are encoded by organelle genomes rather than nuclear DNA. Cytoplasmic male sterility (CMS), a maternally inherited trait that inhibits pollen production, is of particular utility in hybrid seed production. CMS has been moved across species through protoplast fusion, leading to new sources of male sterility useful for hybrid breeding programs. Another example involves the herbicide resistance traits encoded by chloroplast genes, which have been transferred among species leading to new choices for weed control in agricultural systems. Protoplast fusion is a significant tool for metabolic engineering research, as it allows the gain of biosynthetic pathway of another species, which could lead to the synthesis of new compounds or the improved production of valuable metabolites. As an example, one avenue of this approach has been to use the fusion of medicinal plant cells possessing varying biosynthetic potential to generate new or altered bioactive compounds. In a similar fashion, microalgae species featuring compatible metabolic pathways have been explored for improving biofuel production efficiency. Applications of protoplast fusion also address fundamental studies of genome organization, gene expression and nuclear-cytoplasmic interactions. Somatic hybrids and cybrids (cells containing the nucleus of one species and the cytoplasm of another) are valuable experimental systems from which we can learn how the nuclear and organelle genomes interact to coordinate cellular functions. Armed with a better understanding of genome evolution, cellular regulation reflected through patterns of gene expression, chromosome elimination, and organelle segregation found in fusion products will yield insight into other system-level aspects of cell regulations. Traditional biotechnology of protoplast fusion has enabled the construction of new expression systems for recombinant proteins and secondary metabolites. Through introduction of biosynthetic pathways or regulatory pathways from other species, scientists have generated cell lines that produce

high amounts of product or new products altogether. Moreover, protoplast fusion is utilized to transfer new genetic material into species that are not amenable to other transformation methods thus broadening the scope of organisms possible to genetically modified. While it has many applications, protoplast fusion has its own limitations and challenges. The generation of viable plants from fusion products is technically demanding for many species such as cereals and woody perennials. Moreover, the general genomic instability associated with wide hybrids can also result in unpredictable phenotypes and lower the utility of fusion-derived lines in practice. In addition, in some countries protoplast fusion is regarded as a type of genetic alteration under regulatory frameworks which may restrict the commercial introduction of fusion-derived varieties. Particularly, combining protoplast fusion with other cutting-edge breeding and genomic techniques represents a promising route for overcoming some of these limitations in the future. For example, protoplast fusion along with genome editing techniques could allow for the modification of fusion products for improved stability or the insertion of specific traits. Likewise, the use of high-throughput phenotyping and genomic selection methodologies could significantly speed up the discovery and breeding of promising fusion-based lines.

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 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. This instability results from the enforced merging of diverging genomes that may differ in chromosome number, structure, or replication timing. But although this genomic restructuring can at times produce useful variation, more commonly it provides a barrier to the advancement of stable, uniform varieties. This challenge may be countered by combining recent, advanced cytogenetic techniques with genomic strategies to monitor and potentially stabilize hybrid genomes. Asymmetric fusion techniques that transfer portions of one parental genome only could allow for a more controlled delivery of particular traits without the burden of much of Chinese ink mustard genomes. Poor knowledge of factors governing compatibility of fusion partners is further limiting the predictability and efficiency of protoplast fusion. Interactions between the nucleus and the cytoplasm, epigenetic modifications and the molecular mechanisms underlying cell recognition and fusion, are poorly understood, particularly in the context of widespread hybridization. With improvements in genomic and proteomic technologies, more thorough characterization of these factors may allow better prediction of compatible combinations and guide the optimization of allogenic fusion protocols. In addition, the discovery of (1) genes associated with (non)hybridity, which confer the ability to undergo cell fusion; and (2) molecular markers that predict hybrid fitness, could inform selection of parental materials and fusion methods. In practical terms, protoplast fusion protocols are laborious and technically complex, and, therefore, they rarely find routine applications in crop improvement programs. These techniques require sterile conditions, specialized equipment, and skilled personnel, making them inaccessible in resource-limited settings. Well-established protocols and automated systems for protoplast isolation, fusion, and culture would make this technology more accessible and encourage its inclusion in additional breeding initiatives. Moreover, developing a set of reference standards for fusion efficiency assays and characterization of fusion products, would improve comparability between studies and different laboratories.

Another key consideration influencing the use and acceptance of products derived from protoplast fusion is their regulatory status. In some jurisdiction, protoplast fusion-derived crops are classified as genetic modified organism and face further regulatory hurdles and potential on-market prohibitions. This classification is due to the fact that protoplast fusion is considered a much more invasive manipulation of the plant genome than classical breeding methods. Ongoing dialogue between scientists, regulators and stakeholders about the nature and safety of protoplast fusion techniques will be key to developing proportionate, science-based regulatory frameworks that protect innovations while ensuring appropriate safeguards.

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 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-twosey entities to a ubiquitous part of agricultural innovation systems, enabling greener, higher-yielding crops that will sustain the population of the future.

Unit 14: Cybrids

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 perturbations in this relationship might underlie pathological conditions in lower model organisms. Cybrids have applied uses in biotechnology, agriculture, conservation biology, and medicine, making their importance significant beyond basic science. In medical settings, cybrids have been particularly valuable for studying mitochondrial diseases a family of genetically inherited disorders caused by mutations in mitochondrial DNA that disrupt the production of energy by cells. These conditions can present as neurological, muscular ormetabolic disorders and have long been difficult to study, because mitochondria have their own, distinct genetics. Cybrids confer a well-defined experimental environment in which the effects of individual mitochondrial mutations on cellular physiology can be studied against a uniform nuclear genetic background. Cybrids have also paved the way to therapies for mitochondrial diseases. Mitochondrial replacement therapy (MRT) and similar techniques utilize principles of cybrid formation, to generate embryos free from maternal mtDNA mutations and thus potentially prevent the transmission of life-threatening mitochondrial diseases to the next generation. This potential application has already generated significant scientific interest, as well as ethical debates surrounding the genetic modification of humans and human reproduction. In the broader biological sciences, cybrids have provided important insight into species evolution, cellular compatibility, and the coevolution of nuclear and mitochondrial genomes. Generating interspecies cybrids allows the study of the extent of functional compatibility between nuclear and mitochondrial genomes of distantly related evolutionary lineages and can reveal constraints and flexibility of such ancient symbiotic relationships. Exploring cybrid technology further, it is clear that these cellular constructs exist at the intersection of modern biotechnology twine together between fundamental research and applied science, and challenging our definition of cellular identity, compatibility, and species boundaries. As cybrid technology continues to be refined, it should provide even greater insight into how cells work and offer potential game-changer ways to treat diseases that have been resistant to any attempts at cure in the past.

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, ultimately resulting in the generation of mitochondrion-free (rho-zero or ñ0) 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 cytoplast (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 ñ0 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 cytoplast will grow in this selective medium. Conversely, if there are nuclear genes required for cytoplast 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.

This method provides better control over the number and purity of transferred mitochondria but may achieve lower efficiencies than whole-cell fusion strategies. Newer developments in cybrid technology have integrated aspects of somatic cell nuclear transfer (SCNT), or the infamous used of the technique in animal cloning. This is accomplished by taking the nucleus out of an oocyte or zygote and transferring in the nucleus from a somatic cell. If used in conjunction with mitochondrial transfer or replacement this technique could generate cybrids possessing defined nuclear and mitochondrial constituents and hold promise for reproductive medicine applications aimed at avoiding the intergenerational transmission of mitochondrial disease. The efficiency of cybrid formation can be highly variable depending on the specific methods and cell types used. Interspecies cybrid formation is also complicated because of

nuclear and mitochondrial incompatibilities between the two evolutionarily distant species. These incompatibilities often result in compromised mitochondria function, slowed cellular growth, or in extreme cases, a total lack of viability; all of which reflect the co-evolutionary interactions between nuclear and mitochondrial genomes. Nonetheless, interspecies cybrids working with relatively proximate species have thus far proved successful, yielding critical insight regarding evolutionary restrictions and species limitations at the cellular level. After fusion, cybrids go through an adjustment phase where foreign mitochondria interact with the host nuclear genome to reach a new equilibrium. This adjustment may also lead to alteration of the number, morphology, and function of mitochondria, at least during the initial period of the establishment of the new mitochondrial–nuclear relationship. In general, mitochondrial heteroplasmy the state in which multiple mitochondrial genotypes are present within a single cell may exist in recently generated cybrids but usually resolves over time via mitochondrial selection and segregation into homoplasmic cells bearing a single mitochondrial genotype. Cybrids thus provide an interesting opportunity to study the fundamental biology of mitochondria, and have the potential to contribute significantly to mitochondrial research, but the technical complexities of cybrid formation have catalyzed ever-evolving innovations in the methodology to streamline advantages around efficiency, repeatability, and relevance. These advances consist of further refinement of cell fusion techniques, the invention of more effective selection markers, and their combination with other cutting-edge technologies, such as genome editing tools (CRISPR-Cas9), to further modify the nuclear or mitochondrial genomes of cybrids. These methodologies have witnessed steady evolution, with the promise of further augmenting the cross-disciplinary and technologically empowering utility of cybrids. Cybrids are known to establish via a complex process involving membrane fusion, compatibility of nuclear and mitochondrial genomes, and adaptation mechanisms in the host cell that place constraints on the successful establishment of cybrids. These findings not only will enable cybrid formation but also can reveal important features of cellular biology, including the persistence of organelle inheritance, nuclear-mitochondrial communication and the limits of cellular plasticity across species.

Applications of Cybrids

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 — heterogenous 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 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. This technique seeks to stop the transmission of maternal mitochondrial mutations to progeny, and can effectively eradicate the risk of mitochondrial disease across generations. Although this technology has sparked an ethical debate surrounding genetic modification for human reproduction, it could provide hope for couples whose family members have severe mitochondrial diseases and who may otherwise have to choose between a child with potentially devastating consequences for the baby, or not having biological children at all. Mitochondrial transplantation delivering an intact mitochondrion directly into cells with dysfunctional mitochondria is being actively pursued as a potential therapy for acquired mitochondrial dysfunction caused by diseases such as stroke, cardiac ischemia, and neurodegenerative disorders. Specifically, through methods involving cybrid formation principles, this strategy has demonstrated encouraging efficacy in both preclinical and early clinical iterations with a particular promise in targeted acute ischemic injuries due to a distinctly heavy involvement of mitochondrial dysfunction in the tissue damage process. Cybrids also are important tools in pharmacogenomics and drug development, allowing scientists to assess the effects of mitochondrial genetic variation on drug response. Some medications, including various antibiotics, antiviral agents and anti-cancer drugs, can create side effects through their interference with mitochondria function a process called mitochondrial toxicity. By using cybrids that have different mitochondrial genotypes, it is possible to identify mitochondrial genetic risk factors for these reactions, assisting in formulating precise medication regimens that consider both nuclear and mitochondria genetic factors in the individuals.

Cybrids have taught us the fundamental role of mitochondrial dysfunction in senescence in aging space. The so-called “mitochondrial theory of aging” suggests that the accumulation of mtDNA mutations over one’s lifetime plays a major role in aging in

that it first inhibits energy production and secondly increases oxidative stress. Cybrids made using mitochondria from younger compared to older individuals, or with ritually introduced mtDNA mutations facilitate direct testing of this hypothesis and assessment of potential interventions to maintain mitochondrial capacity during life. Cybrids have applications in agriculture and animal husbandry beyond human medicine. These could include its use in livestock breeding where cybrid technology has the potential to speed up genetic improvement by combining desirable nuclear genomes with mitochondria targeted towards particular production traits, as well as in terms of meat quality, milk production and adaptation to environmental stressors. Indeed, in the context of crop improvement, cybrids (or the analogous cytoplasmic hybrids in plants) provide a route to introduce useful cytoplasmic traits, such as herbicide resistance, enhanced photosynthetic efficiency or stress tolerance, without modifying the nuclear genome. Cybrid technology makes for interesting reading in conservation biology as it may allow preservation of genetic diversity and even the resurrection of extinct species. For example, if preserved somatic cells from endangered or recently extinct species were combined with oocytes from closely related species, researchers might obtain embryos that possess the nuclear DNA of the endangered/extinct species but the mitochondria of a closely related living species. Although this method does not maintain the full genetic integrity of the source species (the mitochondria are foreign), it could therefore provide a model for retaining nuclear genetic variation where full conservation is unattainable. Interspecies cybrids those made by integrating nuclear and mitochondrial components from multiple species also are useful tools for evolutionary biology. Creating cybrids between distantly and closely related members of one of the trees, systematically, the boundaries of nuclear-mitochondrial compatibility can be circumscribed, and can also provide insights into the rates of co-evolution of these genetic systems, and the mechanisms involved. These have shown that compatible cybrids can be generated between closely related species but compatibility wanes with evolutionary distance, which is explicable by the cumulative divergence of nuclear-encoded mitochondrial proteins and their interaction with mtDNA-encoded constituents. Cybrids are being used in biotechnology as production platforms for specialized biological products. Cybrids45,45 combining the different growth characteristics or advantages of cultivation of one cell line,

combining them with the product-synthesis capability of another cell line could also provide a route for improved biopharmaceutical production. Also, cybrids with engineered mitochondria could act as living bioreactors, producing desirable metabolites or energy-intensive compounds needed for medicine, industry or environmental remediation. In recent times, principles of cybrids have become absorbed by the emerging area of synthetic biology, whereby whole cells containing designer mitochondrial genomes can be constructed for desired cellular tasks. Still largely theoretical, this approach could one day yield cybrids with innovative metabolic capabilities customized for complaint on a biofuel manufacturing plant to environmental decontamination. New applications are being developed as cybrid technology evolves. Cybrids have been used to study nuclear-mitochondrial crosstalk in cancer progression, to investigate the contribution of mitochondrial dysfunction toward neurodegenerative disease mechanisms, and to explore the therapeutic potential of mitochondrial transfer for diseases defined by bioenergetic failure. But in addition to these promising applications, cybrids also raise significant ethical, regulatory, and safety issues, especially when applied to human reproduction or interspecies applications. Ongoing discussions among scientists, ethicists, and policymakers continue over regarding the status of organisms with mixed genetic heritage, the potential ecological impacts of engineered cybrids, and the appropriate regulatory frameworks for technologies that incorporate cybrid methodologies. Thoughtful consideration of these matters will be important for harnessing the full potential of cybrid technology while ensuring responsible use. Cybrids serve as a powerful tool in biotechnology with applications across a range of fields. And the implications of cybrid technology are vast — from helping clarify our understanding of basic cellular biology to providing hope for families grappling with mitochondrial diseases, cybrid technology has the potential to advance our understanding of life at the cellular level and create new tools for tackling major challenges in medicine, agriculture, conservation, and more. With ongoing investigations in this area, it is likely that the cybrid approach will continue to be an exciting avenue of research with applications both to basic biology and biomedical innovation.

MCQs:

1. **What is the primary goal of protoplast culture?**

8. Discuss the different levels at which gene expression is regulated in eukaryotes.

9. What are transcription factors, and how do they regulate gene expression in eukaryotes?

10. Describe the significance of gene regulation in cellular differentiation and development.

one of these four bases in the genome is swapped out for another one. Single nucleotide polymorphisms (SNPs) occur about once every 300 nucleotides in the human genome, making them the most common type of genetic variation. The implication of SNPs goes well beyond genetic interest. These variations potentially determine an individual's susceptibility to specific diseases, reaction to various medications and even distinct physical and physiological traits. For example, SNPs within genes associated with metabolism may affect an individual's ability to metabolize specific nutrients or drugs, while variations within genes involved in the immune response may affect an individual's susceptibility to an infectious agent.

Regular Indels

Another major class of genetic variation is insertions and deletions. Such alterations are changes in the sequence of nucleotides in the genetic code by insertions or deletions. Indel can be from a single nucleotide to large chunks of DNA, which can lead to large genetic changes. Indels that fall within coding regions of genes can change protein structure and function on a large scale, and thus, have dramatically different potential consequences. Some of the mutations were frameshifts, where a single nucleotide insertion or deletion alters the entire reading frame. This results in specifically different amino acid sequences or in premature stop codons that shorten proteomic synthesis.

Copy Number Variations (CNVs)

b) Cell fusion

c) Protoplast isolation

d) Somatic hybridization

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

a) Cellulase

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

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

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.

Long Questions:

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

2. 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 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.

Unit 15: Transgenic Plants

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 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.

Techniques for Generating Transgenic Plants

Transgenic plants are produced by using complex methods for inserting foreign DNA into plant cells followed by regeneration of whole plants from the transformed cells. Plant transformation methods have been developed with various advantages, limitations, and applicability for different plant species. The selection of method is contingent upon factors such as plant species, the type of tissue utilized, transformation efficiency desired, and available resources.

Precursor for Agrobacterium Transformation

This technique is limited amongst monocots, but Agrobacterium tumefaciens can still play a role in the development of genetic transformation of many plant tissues, particularly for dicots, making it one of the most commonly used systems for producing transgenic plants. This technique takes advantage of the natural propensity of a soil bacterium called A. tumefaciens, which when infecting plants, transfers a piece of its DNA (the T-DNA) into the plant cell itself. In nature, the transferred genes code for enzymes involved in the biosynthesis of plant growth hormones and opines, in which the bacteria can feed; this process results in the formation of crown gall tumors. For this to be used for plant genetic engineering, the genes that induce tumors in the bacterial Ti (Tumor-inducing) plasmid are removed and replaced with the genes of interest and selectable marker genes. The engineered bacteria are next co-cultivated with plant tissue, usually leaf discs, segments of stems, or callus tissue. Following co-

cultivation, the bacteria transfer the modified T-DNA to the plant cells, which carries the genes of interest. The selected cells are regenerated into whole plants. Some advantages of Agrobacterium-mediated transformation are moderate transformation efficiency, a capability for the transfer of large segments of DNA and a generally single or low copy number of the insert. This approach has worked well in many crop plants including tobacco, tomato, potato, and rice. Its application was less effective on monocotyledonous plants (e.g., cereals) instantaneously, which are not permissive to Agrobacterium infection. Progression of these 'super-virulent' Agrobacterium strains, as well as optimization of transformation protocols, has led to the successful transformation of many monocot species as well.

Methods of Producing Transgenic Plants

Direct Gene Transfer Methods

In this review, we summarize the various methods of direct gene transfer techniques — the process of physically or chemically introducing DNA into plant cells without biological vectors. These methods have been extremely useful for plant species that are not transformable via Agrobacterium.

Bombardement Particule (Biolistique)

Particle bombardment, commonly referred to as biolistics or gene gun technology, is a process that coats microscopic gold or tungsten particles with DNA before accelerating them into plant tissue at a very high velocity. The particles are penetrating to the cell walls and membranes and package the DNA into the cytoplasm and nucleus. A portion of the DNA that gets introduced, integrates into the plant genome, leading to stable transformation. This has been especially effective for monocot species such as corn, wheat, and rice, which were once resistant to transformation using agrobacterium. Particle bombardment enables one to deliver multiple genes at once and is not limited biologically on what kind of DNA can be delivered. However, it tends to produce multi-copy insertions of the transgene that can become silenced or expressed in an unstable fashion. Moreover, the efficiency of transformation is lower than that of Agrobacterium-mediated methods, and the integration pattern is less predictable, which may disrupt the functions of endogenous genes.

Protoplast Transformation

A protoplast is a plant cell that has 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.

Other Methods

Various alternative techniques have since been developed for plant transformation, such as:

- **Silicon Carbide Fiber Mediated Transformation**: In this process, plant cells are mixed with silicon carbide fibers and DNA, which, by creating microscopic wound in cell walls, allows for DNA to enter.
- **Liposome-mediated transformation:** They are small, artificially prepared vesicles which can encapsulate DNA and fuse with the cellular membrane to release the DNA into the cell.
- **Vacuum Infection (mostly applied for Arabidopsis):** Many protocols have used semi-immersed plant tissue in an Agrobacterium suspension and exerted vacuum (bacterial would enter into plant tissue).

- **Floral Dip:** A less complex approach to vacuum infiltration established that flowering plants could be dipped into Agrobacterium suction, resulting in ovule transformation for transgenic seeds.

Vector Construction

Irrespective of the method of transformation used, the construction of the appropriate vector containing the genes of interest is a vital step in the production of transgenic plants. These vectors usually contain:

- **Gene of Interest:** The gene responsible for the desired trait, as well as appropriate regulatory elements (such as promoters, enhancers, and terminators) to ensure proper expression.

- **Selectable marker genes:** These include genes that confer resistance to antibiotics (including kanamycin/hygromycin) or herbicides (e.g., phosphinothricin), and thus allow the selection of transformed cells.

- **Reporter Genes**: Genes that encode proteins that can easily be detected (e.g. GFP, GUS), and assist in identifying and analyzing transformation events.

Modular cloning systems are commonly used for the assembly of new constructs containing multiple genes. More recently, developments in synthetic biology have allowed for the construction of tailor-made genetic circuits that allow for more finely-tuned control of gene expression.

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 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 (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.

In addition to selectable markers, reporter genes are frequently employed to determine transformation and/or visualize transformation. These genes code for identifiable proteins that can be detected using simple tests. Examples of such reporter genes are:

- **GUS (â-glucuronidase):** This enzyme is capable of cleaving the substrate X-gluc and gives a blue color. When incubated with X-gluc, GUS-expressing tissues turn blue, allowing gene expression to be visually assessed.
- **GFP (Green Fluorescent Protein):** This protein fluoresces green when excited with blue or UV light, providing non-destructive visualization of gene expression.
- **Luciferase**: Key to a light-generating reaction whose product can be detected with specialized imaging hardware.

Examples of reporter genes include â-glucuronidase (GUS) or green fluorescent protein (GFP), and they can be used for several applications such as optimizing transformation procedures, analyzing gene expression, or confirming transgene expression in various plant tissues.

Selection Strategy

Usually, you get the transforming tissue cultured on the media with the selective agent (antibiotic, herbicide, etc...) Non-transformed cells die or stop growing over time, whereas transformed cells survive and proliferate forming either callus tissue or developing directly into shoots or embryos, depending on the culture conditions and plant species. Selective agent concentration needs to be optimized in order to kill non-transformed cells without excess toxicity to transformed cells. Using too low of a concentration might allow non-transform cells to survive while too high a concentration might prevent the transformed cells from growing.

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**: 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.

Transformation and Regeneration of Transgenic Plants

Then cells that have been transformed must be regenerated into whole plants. Plant cells are totipotent a single cell can grow into an entire plant when conditions are favorable. However, the high-throughput application of this feature is truly species and tissue dependent.

Regeneration Methods

There are two main pathways for plant regeneration:

- **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.

What Are the Factors That Affect Regeneration

Plant regeneration efficiency is affected by some different factors.

- **Genotype**: Genotypes or varieties of a given species can show wide ranges in regeneration potential. This is a significant barrier in developing transformation protocols for recalcitrant species or varieties.

- **Explant Type and Age:** The type of tissue used for transformation and its developmental stage (e.g. leaf discs, cotyledons, immature embryos) can significantly influence regeneration. Regeneration potential is overall greater in younger, actively dividing tissues.

- **Culture Medium Components:** Supplemental components of the growth medium including nutrients, vitamins, plant growth regulators, and other additives

are critical driver of cell growth and development. The ideal medium compositions differ depending on species and regeneration types.

- **Environmental Conditions:** The light intensity, photoperiod, temperature, and humidity of the culture environment play vital roles in regeneration success.
- **Time in Tissue Culture:** Extended tissue culture periods may induce somaclonal variation (genetic/epigenetic changes) and reduced regeneration potential. In general, the less time in culture, the better to preserve potential genetic deviations.

Challenges in Regeneration

There can be several challenges faced during the regeneration of transgenic plants:

- **Recalcitrance:** Certain plant species or cultivars are intrinsically difficult to regenerate from tissue culture. This is especially true for a number of economically important crops, including both cereals and legumes. However, extensive optimization of regeneration protocols or use of more responsive genotypes is often required to overcome recalcitrance.
- **Somaclonal Variation:** During tissue culture, genetic and epigenetic changes can happen, resulting in phenotypic variation among the regenerated plantations. The variation can influence the expression of the transgene or the performance of the plant overall. Potential solutions against somaclonal variation include minimizing time in culture and optimizing culture conditions.
- **Chimerism:** Some of the cells in a particular tissue or callus might not be transformed during transformation. If there is regeneration from a mixture of the transformed cells and the non-transformed ones, then the resulting plant could be chimeric, where some tissues express the transgene and some do not. This may result in unstable transgene expression or loss of transgene in the coming generations.
- **Transgene Silencing:** Over the integration of multiple copies of the same transgene or its insertion into specific genomic areas, the gene silencing systems

can become activated and inactivate both transgene expression and expression levels.

Characterisation and Evaluation Of Transgenic Plants

Transgenic plants need to be characterized in depth following regeneration to confirm the transgene integration and expression. This characterization usually includes:

- **Understanding the Genetics:** Methods like PCR and Southern blotting are employed to verify that the transgene is indeed present, and to determine its integration pattern in the plant genome.
- **Expression Assessment:** RT-PCR, Northern blotting, Western blotting, and immunoassays are used to confirm transgene expression at the RNA and protein level.
- **Phenotypic Analysis:** The targeted phenotypic trait is assessed in the transgenic plants, as well as other phenotypic effects that may not have been anticipated at the start of the study.
- **Transgene Inheritance Assessment:** Transgene segregation and stability are analysed in the progeny by examining segregation and stability in subsequent generations

Once a transgenic line is characterized, promising ones may enter a development pipeline including evaluation under field conditions and testing for safe use in the environment prior to regulatory approval as well as to breed the transgene into high yielding stocks.

New Strategies and Future Directions

Specifically, the techniques for plant transformation and regeneration remain an ever-evolving area for improving transformation efficiency, precision, and functionality in a wide array of plant species. Here are some emergent approaches:

- **Developmental Regulators:** Developmentally regulatory genes that control processes such as embryogenesis or the formation of specific organs have already

been used successfully to improve regeneration capacity, especially in recalcitrant species, e.g., BABY BOOM (BBM) and WUSCHEL (WUS).

- **Genome Editing in Planta:** Approaches for in planta genome editing, including means of delivering genome editing components directly to seeds or meristems, are being developed (Tzfadia et al., 2021) to circumvent regeneration bottlenecks.
- **Single-Cell Approaches**: Development of technologies for isolating, manipulating, and regenerating single cells of plant systems offers exciting new possibilities for precision genetic modification that reduces chimeric analysis.
- **Systems Biology:** Utilizing genomics, transcriptomics, proteomics, and metabolomics data to achieve a global perspective of regeneration, with the promise of underpinning more rational strategies to improve regeneration efficiency.

These new advances hold the potential to broaden the spectrum of plant species that can be genetically manipulated, and to increase the accuracy and efficiency of transgenic plant generation, thereby aiding the generation of crops with superior attributes related to agriculture, nutrition and environmental sustainability. To summarize, selection and regeneration are key features in the generation of the transgenic plants. Although impressive advancements have been made in establishing and optimizing these procedures in various plant species, there are still some species that are recalcitrant (hard-to-transform) as well as aspects such as precise, stable transgene expression which still require extensive work. Innovations are needed to give full potential of plant genetic engineering to solve global challenges in agriculture, nutrition, and environmental sustainability.

Unit 16: Techniques of Transformation

Plant transformation represents one of the most significant developments in modern plant biotechnology, enabling the introduction of foreign genes into plant genomes to confer novel traits or study gene function. The ability to manipulate plant genomes has revolutionized both fundamental plant research and applied agricultural biotechnology. This MODULE explores the primary techniques employed for plant transformation, including their principles, methodologies, advantages, limitations, and applications.

INTRODUCTION TO THE PRODUCTION OF TRANSGENIC PLANTS

Plant transformation refers to the process of introducing foreign DNA into plant cells followed by integration of the introduced DNA into the plant genome, regeneration of transformed cells, and subsequent expression of introduced genes. Since the first successful plant transformation in the early 1980s, numerous techniques have been developed with varying efficiencies across different plant species. The selection of an appropriate transformation method depends on multiple factors including plant species, tissue type, transformation efficiency requirements, available resources, and the specific research objectives. Several critical components must be considered for successful transformation: the gene delivery system, selectable marker genes for identifying transformed cells, appropriate promoters for gene expression, and efficient plant regeneration systems. Plant transformation has evolved from being a complex laboratory technique to becoming a routine procedure in many laboratories. However, transformation of certain plant species, particularly monocots and woody plants, continues to present significant challenges. This has spurred continuous innovation in transformation methodologies to overcome species-specific barriers and improve transformation efficiencies. The primary transformation techniques can be broadly categorized as biological methods (such as Agrobacterium-mediated transformation) and physical/chemical methods (including electroporation, microinjection, particle bombardment, and polyethylene glycol-mediated transformation). Each method has distinct advantages and limitations, making them suitable for different applications and plant species. Beyond the method of DNA delivery, successful transformation requires careful consideration of molecular components including selectable markers (such as antibiotic or herbicide resistance genes), reporter genes (like GUS or GFP for visual confirmation of transformation), promoters (constitutive, tissue-specific, or inducible), and appropriate terminator sequences. The molecular design of the transformation vector significantly impacts the efficiency and stability of transformation and subsequent gene expression. Recent advances in transformation technology have focused on developing marker-free systems, improving transformation efficiencies, expanding the range of transformable species, and developing site-specific integration methods for precise genome modification rather than random integration. The emergence of genome editing technologies like CRISPR/Cas9 has further revolutionized plant transformation, enabling precise modifications to the plant genome without necessarily introducing foreign DNA.

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 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 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 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. Despite these advantages, microinjection has significant limitations

processing 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 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.

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:

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

Methodologies for Agrobacterium-Mediated Transformation

Several methodologies have been developed for Agrobacterium-mediated transformation, each optimized for different plant species, tissue types, and research objectives. The main approaches include:

Leaf Disc Transformation

Leaf disc transformation was one of the earliest methods developed and remains widely used for dicotyledonous plants like tobacco, tomato, and potato. The procedure involves:

1. Cutting leaf tissue into small discs (typically 0.5-1 cm diameter)
2. Co-cultivating the discs with Agrobacterium suspension
3. Transferring the discs to selection medium containing antibiotics to eliminate Agrobacterium and select for transformed plant cells
4. Culturing the discs on regeneration medium to induce shoot formation from transformed cells
5. Rooting the regenerated shoots to obtain complete transformed plants

This method is relatively simple and efficient for many dicot species but is less suitable for monocots and woody plants.

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 Planta 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:** 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

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:

- **Agrobacterium strain:** Different strains vary in their virulence and host range
- **Bacterial density:** Typically OD600 between 0.1-1.0, depending on the system
- **Co-cultivation duration:** Usually 1-3 days, balancing transformation efficiency against overgrowth of bacteria
- **Co-cultivation medium:** Often supplemented with acetosyringone to induce vir genes
- **Temperature:** Typically 22-28°C, with lower temperatures sometimes favoring T-DNA transfer
- **pH:** Usually slightly acidic (5.2-5.8) to enhance vir gene induction
- Light conditions: Dark or low light conditions often enhance transformation

After co-cultivation, antibiotics are added to eliminate Agrobacterium, and selective agents are applied to identify transformed plant cells, which are then regenerated into whole plants.

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:** These influence both susceptibility to infection and regeneration capacity

- **Plant defense responses:** Some plants mount strong defense responses against Agrobacterium, reducing transformation efficiency

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:** 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

- **Vector backbone:** Some vector backbones are more efficient for specific applications
- **Promoter choice**: Affects expression levels of transgenes and selectable markers

Optimization of these factors requires a systematic approach and often involves significant trial and error for new plant species or genotypes. Recent advances in understanding the molecular mechanisms of Agrobacterium infection have led to more rational approaches to improving transformation efficiency.

Applications and Recent Advances

Agrobacterium-mediated transformation has become an indispensable tool in plant biotechnology with applications ranging from basic research to commercial crop improvement:

Functional Genomics

- Gene function analysis through overexpression, gene silencing, or knockout
- Promoter analysis using reporter genes
- Protein localization studies using fluorescent protein fusions
- Study of developmental processes through tissue-specific or inducible gene expression

Crop Improvement

- Development of insect-resistant crops through expression of Bt toxins
- Creation of herbicide-resistant varieties for improved weed management
- Engineering disease resistance against viral, bacterial, and fungal pathogens
- Biofortification of crops with enhanced nutritional content
- Improvement of abiotic stress tolerance (drought, salinity, heat)
- Modification of plant architecture, flowering time, and yield components

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 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

- Site-specific recombination systems (Cre/lox, FLP/FRT) for marker excision
- Transposon-based systems for marker removal
- Visual markers replacing antibiotic or herbicide resistance genes
- Direct selection of transformants based on transgene function

Integration with Genome Editing

The combination of Agrobacterium-mediated transformation with CRISPR/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

issues, and regulatory considerations for commercial applications. Ongoing research continues to address these challenges, further expanding the utility of this remarkably versatile transformation method.

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 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:

1. 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

5. Integration of transformation techniques with high-throughput phenotyping and multi-omics approaches for more comprehensive evaluation of transformants

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

As these advancements continue, plant transformation will likely become more efficient, precise, and widely applicable across diverse plant species. This progress will not only enhance our fundamental understanding of plant biology but also accelerate the development of improved crops to address global challenges in food security, climate change adaptation, and sustainable agriculture. The integration of transformation techniques with genome editing technologies represents a particularly promising direction, potentially allowing precise modifications that mimic natural variation rather than introducing foreign genes. This approach may help address some of the regulatory and public acceptance issues that have limited the adoption of transgenic crops in certain regions. In conclusion, plant transformation techniques have come a long way since their development and continue to evolve rapidly. The ongoing refinement of existing methods and development of new approaches will further expand the toolbox available to plant scientists and breeders, ultimately

contributing to both fundamental plant research and practical applications in agriculture and biotechnology.

Unit 17: 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 become 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 juvenile 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 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 demand 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 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:** 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 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.

Production of Haploids and Double Haploids

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 dotsDH 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 cultivar selection from 10-12 years down to 5-7 years. Likewise, a substantial

proportion of barley varieties bred in developed nations in the last several decades capitalized on DH technology as part of their development pipeline, a testament to the influence of the technique on agricultural production. We have high-throughput sequence analysis available and the capacity to produce and screen large numbers of mutants. As such, tissue culture systems represent an excellent system to make and select for mutations in a defined and controlled milieu. In vitro approaches present multiple advantages over whole-plant mutagenesis:

- Increased mutation rate by introduction of single cells or small tissue clusters
- The capacity to exert selection pressure directly on the cultured cells
- Chimeric structures are eliminated, as in general, each regenerated plan is derived from one cell

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 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 us 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 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 adoption
- it enables the repatriation of germplasm of native crop varieties from foreign gene banks back into their native countries.

The International Potato Center (CIP) and the International Institute of Tropical Agriculture (IITA), for example, routinely distribute improved germplasm through such in vitro methods to national agricultural research systems around the world. This strategy has proven especially useful for vegetatively propagated trees, such as cassava, yam and potato, which presented serious quarantine hurdles if grown from seed.

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 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.

In cotton, embryo rescue–assisted interspecific hybridization has united the best fiber quality of Gossypium barbadense with the yield potential of G. hirsutum (service root reduction method), advancing the commercial exploitation of hybrid potential. In addition to disease resistance, wide hybridization has played a role in developing stress-tolerant cereal crops (e.g., triticale, a wheat × rye hybrid) and interspecific rice hybrids adapted to various stress conditions. With the stressors of climate change only increasing over time, the genetic diversity that can be accessed through embryo rescue will be more important than ever for agricultural sustainability.

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 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:

- 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 trifoliate 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. The somatic hybrids themselves may or may

not be directly commercialized, but they represent valuable new germplasm in a conventional breeding program.

Classifier for Drought Stress Tolerance

Tissue culture systems allow high-throughput screening for stress tolerance in controlled environment, resulting in the faster identification of resistant genotypes. In vitro several abiotic stresses are able to be simulated:

- NaCl or other ions added in culture media as salinity stress
- Osmotic agents such as polyethylene glycol or mannitol to induce drought stress
- For example, temperature stress via high temperatures or low temperatures exposure on cultures

Oxidative stress via chemical inducers such as paraquat

Cells or tissues escaping from these selective criteria can be regenerated to whole plants, revealing, in general, stress tolerance similar to that found in vitro. The approach has produced salt-tolerant varieties of rice, sugarcane and other species, as well crops that are more resilient to drought, cold and heavy metals. In vitro selection is becoming more efficient, as our understanding of stress physiology is deepened and more sophisticated in vitro models with high predictive value are developed.

Synthetic Seed Technology

Synthetic or artificial seed technology extends the concept of somatic embryogenesis to produce propagules that functionally resemble natural seeds. The encapsulation of somatic embryos (or other micropropagules) in a protective gel matrix (often supplemented with nutrients, growth regulators and/or antimicrobial compounds) has led to the development of propagation units able to be handled, stored and planted with standard equipment.

Synthetic seeds have the following advantages in an agricultural sector:

- Propagation of elite hybrids through clones using existing seed planting system

- Longer storage potential than bare somatic embryos
- Defense against mechanical damage and dryness while being taken care of
- The possibility of sowing directly into field or greenhouse conditions
- The incorporation of beneficial microorganisms or crop protection agents inside the encapsulation matrix

The most widespread commercial deployment of synthetic seed technology is in forestry, particularly conifers, where this method has been optimized and scaled by companies such as Weyerhaeuser and CellFor for the establishment of industrial plantations. In the agricultural realm, newly researched synthetic seeds with improved germination rates and field performance have been developed for crops such as alfalfa, carrot, rice, and sugarcane.

The technology shows particular promise for:

- Vegetatively propagated crops where true seed is not available or does not retain desirable characteristics
- Hybrids with costly or complex seed production systems
- Plants with undesirable seed storage attributes

Apart from the aforementioned production of disease-free planting material, tissue culture is the backbone of the clean stock certification programs of many agricultural crops. These programs combine meristem culture with strict indexing procedures in order to provide propagation material with specific phytosanitary standards.

For vegetatively propagated crops such as potato, sugarcane, cassava and sweet potato clean stock programs have:

- Used to remove viral and bacterial pathogens, resulting in a 20-40% yield increase
- Enhanced quality parameters like starch content, sugar concentration, or processing characteristics
- Improved the productivity of seed multiplication systems

Allowed for the international exchange of enhanced varieties

The economic payoffs from those programs are significant. Research in Kenya and Uganda showed benefit-cost ratios of 20:1 to 25:1 for investments in clean seed systems for vegetatively propagated crops based on tissue culture, driven by both yield increases and reductions in pesticide use. In many of the developed nations' certified crop industries, the relevant certification standard now specify tissue culture as the primary propagation method required to produce pathogen-free planting material, acknowledging not only its importance for productivity, but also sustainability in agricultural systems.

Cryobanking of Agricultural Germplasm

Cryopreservation the storage of biological material at ultra-low temperatures, generally in liquid nitrogen ("196 °C) is the most reliable long-term conservation method for vegetatively propagated plants and species with recalcitrant seeds. At these temperatures, all metabolic processes essentially stop, enabling theoretically unlimited storage without genetic drift or physiological damage.

Different cryopreservation techniques have been devised for agricultural germplasm:

- Vitrification, the process by which cells undergo exposure to concentrated cryoprotective solutions, preventing the formation of ice crystals
- Inclusions-dehydration, a combination of sodium alginate encapsulation and local dehydration
- Droplet-vitrification, which overcomes these challenges by incorporating the benefits of vitrification and efficient heat extraction in microdroplets
- Aluminum plates used to expeditiously support vitrified samples are known as V-cryoplates

This has been employed and successfully applied to conserve genetic resources of banana, cassava, potato, sweet potato, yam, and a number of tree fruit species, Moji Shitash, personal communication. Cryobanking was established as a complementary strategy in major international genebanks, such as those operated by CGIAR centres,

and is now utilized alongside field collections and conventional seed storage. The FAO Genebank Standards also consider cryopreservation an integral part of a holistic conservation strategy, especially for vegetatively propagated crops and those with recalcitrant seeds, typical of many tropical staples that are vital for food security in developing areas.

DIscussion: Usefulness of Identified Doubled Haploid Lines

Doubled haploid (DH) line production via anther, microspore or ovule culture procedures is now a routine element of breeding programs for many crops of agriculture importance. This approach drastically speeds up cultivar development by collapsing multiple generations of inbreeding in a single step while also providing completely homozygous lines for studies of genetics and hybrid seed production.

Many crops have already optimized the technical protocols of DH production:

- In cereals, microspore culture is the technique of choice, especially for barley, wheat, rice and maize.
- Microspore embryogenesis is routinely used in commercial brassica (canola, cabbage, broccoli) breeding programs
- Anther culture still stands out as the most commonly used method in Solanaceous crops, such as pepper and eggplant.
- Alternative methods such as ovary co-culture, stress pretreatments and targeted media formulation have improved efficiency for recalcitrant species.

The bandwidth for DH technology is not only to save time but also to change the breeding strategy. Rapid production and phenotyping of large numbers of homozygous lines have allowed for testing on a much larger scale than was feasible in earlier breeding programs, improving the efficiency of selection and enabling faster genetic gain. In hybrid breeding programs, DH technology has also made it possible to exploit heterosis more systematically, in that it facilitates the efficient evaluation of specific combining ability in a broader array of potential parental lines. This helps both to increase yields in hybrid crops and reduce resource requirements for inbred line development.

For crops which are susceptible to systemic viral infection, programs in plant tissue culture based nuclear stock offer a systematic mechanism for storing and distributing pathogen free propagative materials. These programs are generally characterized by:

- Production of virus-free plants at first by meristem culture usually combined with thermotherapy
- Molecular, serological, and biological assaying to rigorously test for pathogen clearance
- Controlled multiplication, staged and regularly tested

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 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 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

Genetic modification of cultured cells via direct transformation

These methods have led to the commercial development of crop varieties with beneficial attributes:

- Tolerant rice lines to salinity and drought
- Callus-induced disease-resistant cultivars of sugarcane screened against the toxins.
- In vitro selection of herbicide-resistant crop varieties
- Varieties with improved quality with modified profiles of secondary metabolites

Despite the heightened focus on genetic engineering, these "conventional" tissue culture approaches continue to provide measurable contributions to crop improvement in cases (for example in specific regions or crops) where transgenic approaches are not readily viable owing to regulatory hurdles or lack of market acceptance.

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.

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

- In pepper and eggplant, new CMS types have been introduced by interspecific somatic hybrids

The economic implications of these developments reach beyond the hybrid seed industry to farmers who are able to exploit the yield advantages of hybrids. For example, the improvement of spermatic hybridization of cytoplasmic male sterility (CMS) systems led the development of more efficient CMS systems supported the expansion of hybrid rice cultivation, indirectly contributing to food security in many parts of Asia.

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
- 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

Use Cases: Commercial Applications

- Glucocerebrosidase (Gaucher diseasetreatment) produced in carrot cell cultures
- Monoclonal antibodies from tobacco cell cultures
- Several of the industrial enzymes derived from the bioreactors of plant cells
- Cosmetic and nutraceutical specialty compounds

As the regulatory frameworks for plant-made pharmaceuticals evolve, this application of tissue culture is expected to grow, providing sustainable production systems for a wider variety of high-value biomolecules.

Economic and Social Impact

Commercial Micropropagation Enterprises

Plant tissue culture has one of the most immediate economical impacts at the global commercial micropropagation scale. This sector includes commercial labs that grow millions of plants each year for agriculture, horticulture and forestry.

The industry structure differs by region:

- Laboratories in North America and Europe only specialize in Exotic Plant Pathogens for high-value ornamentals and fruit crops
- Government-managed facilities in Asia typically favor staple food crops and plantation species
- A variety of public and private laboratories focus on both food security and export-oriented crops in Africa and Latin America

Small operations (producing thousands of plants per year) or industrial (millions of plants per year) operations scale. In only the Netherlands, over 500 million micropropagated plants are produced each year (mostly for the ornamental sector). Over 200 commercial tissue culture companies producing about 500 million plants per year in India.

The economic value chain is far wider than just the laboratories themselves, including:

- Specialty equipment, chemicals, and consumables suppliers

- QC and Certification services
- Nurseries for hardening and acclimatization
- Plant Distribution for Tissue-Cultured Plants
- Technical advisory and training services

Another significant impact is the generation of employment, as the industry provides skilled jobs for rural and peri-urban people. CBPS plant propagation facility at site A in Nyanja can supply 4.5 million plants (Fig 1, lower right corner) per year in a controlled environment, and the outcome of this propagation effort represents more value into the plant (20-50 technicians in a medium sized commercial laboratory between 2-5 million plants /year in a controlled environment).

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 commercializing research innovations

· Public-private collaborations where government facilities forward nuclear stockpiles to private multipliers

These ventures provide jobs, especially for women and youth, who are typically overrepresented in the workforce in tissue culture labs as culture work tends to be precision-focused.

Unit 18: 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. Edible vaccines have come a long way over the decades and many candidates have already moved on to clinical testing. Two well-known examples are the plant-based

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 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 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 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 particularly beneficial for global health initiatives due to their economic potential.

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 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 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..

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?

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

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.

Long Questions:

1. Explain the methods used to produce transgenic plants and their applications.
2. Discuss the role of Agrobacterium-mediated transformation in plant genetic modification.
3. Describe the process and benefits of edible vaccines.

4. Explain the different techniques of transformation and their use in producing transgenic plants.
5. Discuss the advantages and challenges of using transgenic plants in agriculture.
6. How do transgenic plants help in addressing global food security?
7. Describe the process of selection and regeneration of transgenic plants.
8. Explain how edible vaccines are produced and their potential impact on global health.
9. Discuss the role of genetic transformation in creating pest-resistant plants.
10. What are the ethical concerns surrounding the production and use of transgenic plants?

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