Elements of Plant Biotechnology

Dr. Sudip Das

Assistant Professor cum Jr. Scientist,
Department of Plant Physiology and Biochemistry,
Bihar Agricultural University, Sabour, Bhagalpur -813210

Dr. Narendra Kumar

Assistant Professor, Department of Livestock Production Management
(LPM) College of Veterinary and Animal Sciences, Banda University of
Agriculture & Technology Banda U.P.

Dr. R.Pushpa

Assistant Professor in Plant Breeding and Genetics at Tamil Nadu Rice Research Institute,
Tamil Nadu Agricultural University.

Bikram Pradhan

Lecturer In Botany, Department of Biology,
Bharat Institute of Science and Technology,
Bhubaneswar, Odisha, India.

Alphine Publications
Pvt Ltd

Alphine Publications
Pvt. Ltd

Alphine Publications
Pvt Ltd

Published By
Alphine Publications Pvt. Ltd.,
22 Maruti City Extension, Shamshabad Road, Agra–282001
(Uttar Pradesh)
Mo:- 9058586680, 9557613322
Email : alphinepublication@gmail.com
Website: www.alphinepublication.com

Elements of Plant Biotechnology
Dr. Sudip Das/ Dr. Narendra Kumar/ Dr. R.Pushpa/ Bikram Pradhan

I.S.B.N : 978-81-988262-5-1
I^st Edition 2025
PRICE: 650.00

Printed in India
Published by Alphine Publications Pvt. Ltd., Agra.
Laser Typesetting at : izmatgraphics@gmail.com
Printed by D.K. Fine Art Press Pvt. Ltd. New Delhi-110052.

PREFACE

The first edition of Elements of Plant Biotechnology focuses on the various ways that contemporary technologies are applied in various plant biotechnology fields. Numerous fields that are either directly or indirectly related to human civilization are developed with the use of these techniques. The agricultural sector, which develops new and improved crop varieties, is where plant biotechnology is most actively used. This paper explains methods such as synthetic seeds, rDNA technology, plant tissue culture, and many more. The book's main goal is to give readers a wealth of knowledge on all the plant biotechnology methods now employed for crop improvement and plant breeding. These methods will soon see a resurgence due to the present generation's increasing food needs. In addition to providing food for the expanding population, these revolutions will also cure the current generation's physical and mental ailments.

Index

Chapter-1

Scope of Plant Biotechnology: Plant Tissue Culture, Organ Culture & Embryo Culture.

Rajashree Kar[1], Bikram Pradhan[2*] and Dr. R. Pushpa[3]

[1]Department of Science, Sri Aurobindo Integral Education Centre, Sahid Nagar, Bhubaneswar, Odisha, India.
[2]Department of Biology, Bharat Institute of Science and Technology, Bhubaneswar, Odisha, India.*
[3]Plant Breeding and Genetics, Tamil Nadu Rice Research Institute, Aduthurai, Thanjavur District, Tamil Nadu, India.
sudhams99@gmail.com

ABSTRACT

Plant biotechnology refers to a scientific discipline that utilizes technological and biological methods to alter plants for particular aims. It integrates knowledge from plant biology, genetics, molecular biology, and biochemistry to improve crops, create new plant varieties, and enhance agricultural sustainability. Crops that are genetically modified organisms (GMO crops) are agricultural plants whose DNA has been altered through genetic engineering methods. It is aimed to add new traits that the species does not naturally possess. Genetically modified plants known as transgenic plants contain a gene or genes that have been artificially introduced from another organism (often from a different species) to provide the plant with new characteristics not naturally present in that species.

KEYWORDS: Biotechnology, Plant Biotechnology, Scope, Plant Tissue Culture (PTC), Organ culture, Embryo culture.

INTRODUCTION

While multiple definitions of plant biotechnology exist, it is most often viewed as the genetic engineering of plants through recombinant DNA. A variety of scientific methods and techniques for screening and genetically manipulating plants to create beneficial or useful plant/plant products are included in Plant Biotechnology. Plant biotechnology involves using genetic engineering and tissue culture techniques to develop genetically modified plants that possess new or improved desirable characteristics. Among the desirable traits are improved yields and quality, as well as enhanced resistance to negative influences like diseases, pests, and harsh environmental conditions such as freezing temperatures, droughts, and salinity. With plant biotechnology, it is possible to produce in plants useful proteins that are encoded by genes from animals, humans, or microbes. It has been demonstrated by plant biotechnology that all of these objectives can be achieved, at least for the types of plants on which attempts have been made. Scientific techniques that can be used to create cellular- and molecular-based technologies aimed at enhancing plant productivity by improving the quality of plant products and minimizing environmental constraints on plant productivity are encompassed by plant biotechnology. With the help of plant biotechnology, breeders can make precise genetic alterations to introduce valuable traits into plants, exceeding all prior expectations.

PLANT BIOTECHNOLOGY

A variety of scientific methods and techniques for screening and genetically manipulating plants to create beneficial or useful plant/plant products are included in Plant Biotechnology. The

effectiveness of these tools and techniques could be enhanced by nanotechnology. In the near future, it is expected that plant biotechnology will play a crucial role in the increasing crop production. Mineral assimilation enhancement must address the challenges of reducing fertilizer use in developed countries, environmental conservation, sustainable agricultural practices, and the creation of low-input, high-performance crops in regions where soil infertility hinders productivity.

MAJOR MILESTONES

Plant biotechnology is fundamentally based on the concepts of cellular totipotency and genetic transformation. These concepts originate from the Cell Theory proposed by Matthias Jakob Schleiden and Theodor Schwann, and from Frederick Griffith's discovery of genetic transformation in bacteria, respectively.

- In the 1830s and 1840s, Schleiden and Schwann proposed the Cell Theory, which established the cell as the basic unit of life.
- This concept, along with Haberlandt's demonstration of plant cell totipotency in 1902, laid the groundwork for plant tissue culture and later genetic engineering.
- During 1960s, after decades of work, Norman Borlaug creates dwarf wheat that increases yields by 70 percent, launching the Green Revolution that helps save millions of lives.
- In the 1970s, recombinant DNA (rDNA) technologies were developed (Stanford University and the University of California) which opened up new possibilities for genetic engineering in plants.
- In 1980s, the first transgenic plants were developed by inserting bacterial genes into tobacco.
- Golden Rice, a genetically engineered crop designed to generate beta-carotene and combat vitamin A deficiency, was created in 1999.

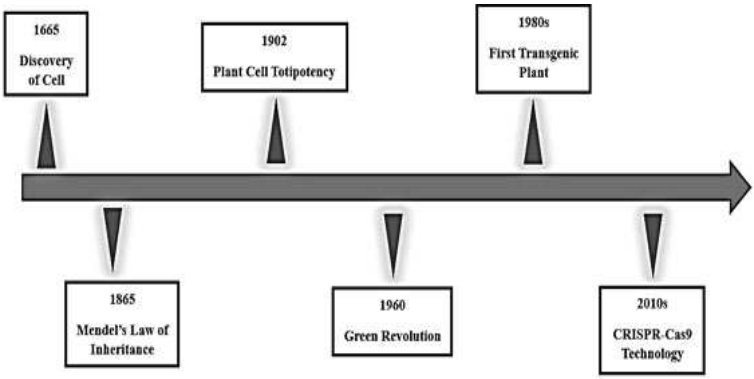

Figure 1. The flow chart represents the important discoveries and inventions which has greatly contributed toward the development of plant biotechnology.

SCOPE OF PLANT BIOTECHNOLOGY

The scope of plant biotechnology is broad and rapidly expanding, with applications in agriculture, industry, environmental management, and health. Here's an overview of its main areas:

1. Agricultural Improvements

- **Crop yield and quality enhancement:** Genetically modified (GM) crops with higher productivity, better nutritional content, and improved shelf life.
- **Pest and disease resistance:** Development of crops resistant to insects (e.g., Bt cotton), fungi, viruses, and bacteria.
- **Stress tolerance:** Engineering plants to withstand abiotic stresses like drought, salinity, and extreme temperatures.

2. Genetic Engineering

- **Transgenic plants:** Introducing specific genes to confer desired traits (e.g., herbicide resistance).
- **Gene editing tools:** Technologies like CRISPR/Cas9

for precise genetic modifications without introducing foreign DNA.

3. Plant Tissue Culture

- ***In Vitro* propagation**: The rapid clonal multiplication of uniform, disease-free plants.
- **Somaclonal variation:** Generating genetic diversity for breeding.
- **Synthetic seeds**: Somatic embryos are encapsulated for convenient storage and planting.

4. Molecular Breeding

- **Marker-assisted selection (MAS):** Using molecular markers to select desirable traits more efficiently than conventional breeding.
- **Genomic selection**: Forecasting plant performance with genome-wide markers.

5. Biopharmaceuticals and Nutraceuticals

- **Edible vaccines:** Plants engineered to produce vaccine components.
- **Phytochemicals:** Production of medicinal compounds like alkaloids, flavonoids, and essential oils.

6. Biofuels and Industrial Products

- **Bioenergy crops:** Engineering plants for higher biomass and oil production for biofuels.
- **Bioplastics and fibres:** Production of biodegradable plastics and sustainable materials.

7. Environmental Applications

- **Phytoremediation:** Using plants to clean up soil, water, and air contaminants.
- **Carbon sequestration:** Enhancing plants' ability to capture atmospheric CO, .

8. Conservation and Biodiversity

- **Cryopreservation:** Long-term storage of plant

germplasm.

- **In vitro conservation:** Maintaining rare and endangered plant species in controlled conditions.

PLANT TISSUE CULTURE

New varieties were traditionally developed using seed propagation. But at present, plantlets generated via micropropagation provide a viable option for numerous plant species. Even though micropropagation demands considerable effort, it reduces the time needed to commercialize new varieties and enables the production of disease-free plants. The contemporary field of plant biotechnology has emerged in a new era of science and technology, where the generation of secondary metabolites, significant enhancements to plant genetics, conservation of germplasm, and development of numerous disease-free and novel varieties are prioritized. The current research in plant tissue culture science includes the production of artificial seeds, biopharmaceuticals, recombinant or other therapeutic proteins, transgenic plants, and plant-made vaccines or antibodies (plantibodies).

ORGAN CULTURE

Organ culture refers to the cultivation of organs or plant parts in artificial media or a culture derived from isolated medium. Explants in organ culture can be any part of the plant, such as the shoot (for shoot tip culture), root (for root tip culture), leaf (for leaf culture), and flower (anther, ovary, ovule cultures). Studies examining dependence on growth regulators and other growth factors have demonstrated that organ culture is highly dependable. It also contributes to expanding the scope of advancements in agriculture and horticulture. The main forms of organ culture used for in vitro plant propagation include meristematic culture, shoot tip culture, nodal culture of separate lateral buds, isolated root culture, and embryo culture.

The first organs to be cultured were excised roots. Root tips about 10 mm in length are extracted from young seedlings grown under axenic conditions and placed in an aqueous culture medium, where they generate laterals. The main explant is now divided into sectors, each containing a portion of the main root along with some lateral roots. Each sector is periodically transferred to fresh medium to maintain a continuous supply of roots. Essential substances alone fulfil the medium requirements, yet some cultures show a positive response to the addition of auxin and other growth regulators. Although basic anatomy and metabolism remain intact, the ability to form secondary vascular tissue is gradually lost in cultured roots.

Shoot tip cultures can be classified into two categories: shoot apex culture, where a segment of the shoot tip that includes an apical meristem, a few leaf primordia, and some stem tissue is cultured. Bud can also serve as an explant. For their growth, they require exogenous gibberellin and cytokinin. To foster stem elongation, a medium devoid of exogenous growth regulators is required. Ultimately, culture requires a third medium with auxin for root initiation. The explant may consist solely of an excised and isolated apical meristematic dome, lacking any leaf primordia or stem tissue. Then, they can be cultured either on agar media or on filter paper that is soaked in a liquid medium. The cultures are referred to as apical meristem culture. These cultures necessitate the presence of exogenous hormones in their medium.

Haploid plants are produced from anthers that have been cultured on basal nutrient medium. Anthers are excised from buds chosen at the uninucleate microspore stage, when microspore mother cells are undergoing early meiosis. After sterilization, they are placed on an appropriate culture medium. Haploid plants may be formed directly from pollens in anthers, or a callus may first be created from which plants develop.

ORGAN CULTURE FOR CROP IMPROVEMENT

Organ culture, which is a type of plant tissue culture, entails the in vitro growth of particular plant organs like roots, shoots, or embryos in a sterile and controlled environment. This technique is important for crop improvement as it allows for the propagation of disease-free plants, rapid multiplication of desirable genotypes, and conservation of rare or endangered plant species. Whole plants can be regenerated from explants in organ culture, and these explants can be genetically altered to exhibit traits like pest resistance, drought tolerance, or improved nutritional value. It also facilitates research into plant developmental biology and hybrid production by addressing challenges such as embryo abortion. In summary, organ culture serves as a significant resource in contemporary plant biotechnology, aiding sustainable agriculture and food security.

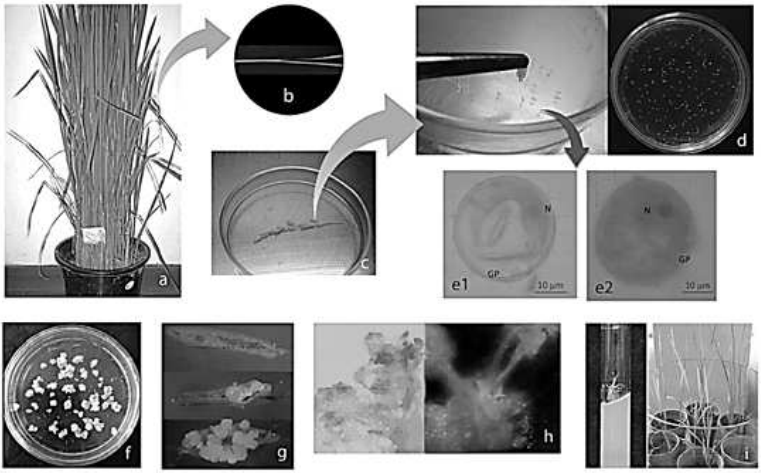

Figure 2. Stages of *in vitro* anther culture for creating doubled haploids in ***indica* rice**. **(a)** Disinfected Mother rice plants **(b)** A panicle **(c)** Surface sterilization **(d)** Anther cultured for callus formation **(e1, e2)** Microspores at uni-nucleate stage; N = nucleus; GP = germ pore; *scale bar = 10 µm* **(f, g)** Calluses development from haploid anthers **(h)** Shoot induction in

anther-derived calluses **(i)** Fully developed haploid plantlets **(Mayakaduwa, & Silva, 2023)**.

EMBRYO CULTURE

Hannig conducted the first embryo cultures in 1904. The seed is cut open to remove the embryo, which is then cultured on a specialized medium. The media requirements differ depending on the species, the stage of the excised embryo, and the experiment's objective. The richness and composition of required media vary among young heterotrophic embryos, globular embryos, and undifferentiated preglobular embryos.

Nucellar tissue removed from ovules (either pre- or post-pollination) and cultured on an appropriate medium first generates a callus, from which numerous tumor-like pseudo bulbils emerge. These bulbils develop into embryos and eventually seedlings. While embryogenesis in the Nucellar tissue of pre-fertilized ovules occurs only with malt extract and adenine, post-fertilization Nucellar tissue from ovules requires casein hydrolase supplementation for proper development.

It is relatively easy to raise mature seeds by culturing ovaries when the embryo is at the globular stage or later in its development. This requires a rather uncomplicated nutritive medium. Seeds from plants with reduced embryos, as well as seeds from parasites, have also been cultured for different purposes. It is quite common to culture ovaries in order to improve fruit quality or study fruit physiology. After pollination, ovaries are cultured. A simple medium containing mineral salts and sugar, sometimes enhanced with vitamins, glycine, and yeast extract, is sufficient. Fruits derived from ovary culture typically have a maximum size that is smaller than that of naturally occurring fruit. Embryo culture techniques are also been implemented for restoration of diversity of many rare and endangered forest plant species.

EMBRYO CULTURE FOR CROP IMPROVEMENT

Embryo culture, a method of plant tissue culture, entails the aseptic cultivation of plant embryos (extracted from seeds) on nutrient media in a controlled lab environment. This technique is particularly useful in enhancing crops because it enables the recovery of embryos from wide crosses that would otherwise not succeed due to post-fertilization obstacles, like embryo abortion. Embryo culture facilitates the transfer of advantageous traits such as disease resistance, stress tolerance, and enhanced yield from wild relatives to cultivated crops by enabling the development of hybrid plants from interspecific or intergeneric crosses. Additionally, it shortens breeding cycles by speeding up seed germination and overcoming seed dormancy. Therefore, embryo culture is essential for increasing genetic diversity and promoting the creation of improved crop varieties.

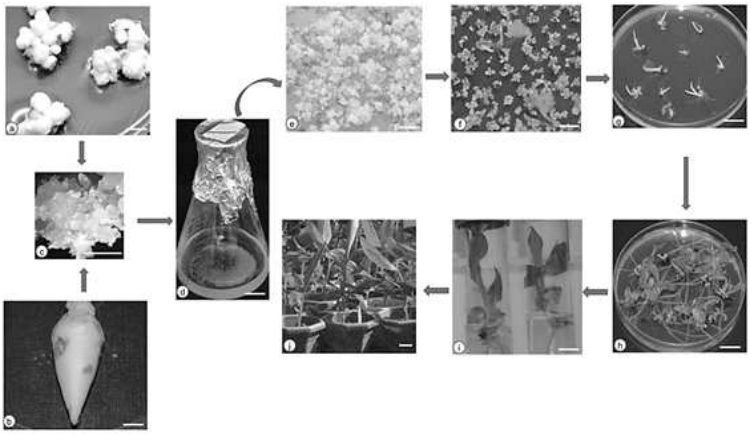

Figure 3. Different stages of somatic embryogenesis and plant regeneration in *Musa* spec. (banana). **(a)** Callus development from multiple meristems explants, **(b)** Male bud, **(c)** Friable embryogenic ideal callus, **(d)** Embryogenic cell suspension, **(e, f, g)** embryo development and maturation, **(h)**

Embryos germinating into plantlets and shoots, **(i)** Fully developed plantlets with roots, **(j)** Complete plants transferred to pots in the greenhouse **(Adero et al., 2023)**.

REFERENCE

- *Adero, M., Tripathi, J.N., & Tripathi, L. (2023). Advances in Somatic Embryogenesis of Banana.* International Journal of Molecular Sciences, *24(13), 10999. https://doi.org/10.3390/ijms241310999*
- *Asati, R., Tripathi, M.K., Tiwari, S., Yadav, R.K., Tripathi, N. (2022) Molecular breeding and drought tolerance in chickpea.* Life, *12, 1846.*
- *Bigini, V., Camerlengo, F., Botticella, E., Sestili, F., Savatin, D.V. (2021). Biotechnological Resources to Increase Disease-Resistance by Improving Plant Immunity: A Sustainable Approach to Save Cereal Crop Production.* Plants, 10, *1146.*
- *Hamdan, M.F., Karlson, C.K.S., Teoh, E.Y., Lau, S.E., Tan, B.C. (2022). Genome Editing for Sustainable Crop Improvement and Mitigation of Biotic and Abiotic Stresses.* Plants, *11, 2625.*
- *Jacob, C., Carrasco, B., Schwember, A.R. (2016). Advances in breeding and biotechnology of legume crops.* Plant Cell Tiss. Organ. Cult., *127, 561–584.*
- *Loyola-Vargas, V.M., Ochoa-Alejo, N. (2024). An Introduction to Plant Cell, Tissue, and Organ Culture: Current Status and Perspectives.* Methods Mol. Biol., *2827:1-13. doi: 10.1007/978-1-0716-3954-2_1. PMID: 38985259.*
- *Mayakaduwa, R., & Silva, T. (2023). Haploid Induction in Indica Rice: Exploring New Opportunities.* Plants, *12(17), 3118. https://doi.org/10.3390/plants12173118*
- *Mmbando, G.S. (2023). The legal aspect of the current use of genetically modified organisms in Kenya, Tanzania, and Uganda.* GM Crops Food, *14, 1–12.*

- *Montilon, V., Susca, L., Potere, O., Roseti, V., Campanale, A., Saponari, A., Montemurro, C., Fanelli, V., Venerito, P., & Bottalico, G. (2022). Embryo Culture,* In Vitro *Propagation, and Molecular Identification for Advanced Olive Breeding Programs.* Horticulturae, *8(1), 36. https://doi.org/10.3390/horticulturae8010036*
- *Pathirana, R., & Carimi, F. (2024). Plant Biotechnology–An Indispensable Tool for Crop Improvement.* Plants, *13(8), 1133. https://doi.org/10.3390/plants13081133*
- *Prasanth, C., Baddigam, K., Kurhade, A. (2024). Embryo Culture and its applications in Biotechnology.* Plant Sciences at a Glance, *269-280. ISBN: 978-93-5834-811-8*
- *Sherpa, R., Devadas, R., Bolbhat, S.N., Nikam, T.D., Penna, S. (2022). Gamma Radiation Induced* In-Vitro *Mutagenesis and Isolation of Mutants for Early Flowering and Phytomorphological Variations in Dendrobium 'Emma White'.* Plants, *11, 3168.*
- *Vasil, I.K. (2008). A history of plant biotechnology: from the Cell Theory of Schleiden and Schwann to biotech crops. Plant Cell Rep. 27(9):1423-40. doi: 10.1007/s00299-008-0571-4. Epub 2008 Jul 9. PMID: 18612644.*
- *Vilcherrez-Atoche, J.A., Silva, J.C., Clarindo, W.R., Mondin, M., Cardoso, J.C. (2023).* In Vitro *Polyploidization of Brassolaeliocattleya Hybrid Orchid.* Plants, *12, 281.*
- *Wieczorek, A.M. & Wright, M.G. (2012). History of Agricultural Biotechnology: How Crop Development has Evolved.* Nature Education Knowledge, *3(10):9.*

Chapter-2

Plant Cell Suspension Culture: Method, Types, Advantages and Limitations.

Soumyajit Nayak[1], Harekrushna Swain[1], Kiran Ku. Bindhani[1], and Dr. Sanjay Ku. Pattnaik[2*]

[1]Department of Biotechnology, NIIS Institute of Information Science and Management, Bhubaneswar, Odisha, India.
[2]Departmentof Botany, Mahendra Institute of Management and Technical Studies, Pitapali, Odisha, India.*
spattnaik1980@gmail.com

ABSTRACT

Cell suspension culture refers to the process of single cells growing more quickly in a liquid media. An orbital shaker is used to continuously stir the liquid medium. Scientists utilize this culturing method to investigate the growth and development of cells. Additionally, several companies use tissue culture to extract specific components from plant cells. The medium's agitation also applies a slight pressure to the tissue, causing it to fragment into single cells and smaller cell fragments. The consistent mobility and dispersion of cells throughout the media are preserved by this agitation. As a result, it is a crucial step in cell suspension culture.

KEYWORDS: Biotechnology, Suspension Culture, Bioreactor, Tissue Culture, Plant Products, Secondary metabolites.

INTRODUCTION

Suspension cultures are typically started by moving pieces of undifferentiated callus to a liquid medium, which is stirred during the culture period. Another method of initiating these

cultures is to inoculate a liquid medium with an explant of differentiated plant material, such as fragments of cotyledon or hypocotyls; however, this method results in longer culture times. A friable callus yields an excellent suspension culture with a high proportion of single cells and tiny cell clusters. A higher auxin:cytokinin ratio can occasionally result in a more friable culture, which improves suspension culture. Periodic subculturing is also necessary for a successful suspension culture. Subculturing is always carried out when the medium's cell density is at its highest. Soon after inoculation, the culture's growth exhibits an initial lag phase before any indication of cell division. An exponential phase follows, during which the number of cells increases exponentially. A linear phase follows shortly after, during which the cell population's growth rate continuously declines. Cell division eventually ceases, and the maximum cell density is reached. This represents the stationary phase.

An alternative method is offered by plant cell culture, which could be appealing in some situations, such as when the source plant is hard to grow, takes a long time to grow, or produces few metabolites; when chemical synthesis has not been accomplished, or when it is technically challenging. The cell culture's metabolite yield may be noticeably higher than that of the parent plant. As a result, this approach allows for the controlled and repeatable production of the metabolite regardless of climate or location.

CELL SUSPENSION CULTURE

In plant biotechnology, plant cell suspension cultures are a common method for investigating a variety of phenomena without having to deal with the structural complexity of the plant organism itself. The high pace of cell development, the enormous amount of material available, the homogeneity of an *in vitro* cell population, and the high reproducibility of circumstances make suspension-cultured cells an excellent

choice for analyzing intricate physiological processes at the cellular and molecular levels. Furthermore, the generation of high-value secondary metabolites and other compounds of commercial relevance can be facilitated by plant cell cultures.

PHASES OF SUSPENSION CULTURE

Selecting a strong parent material, optimizing the surface sterilization process, inducing, maintaining, and mass-propagating the callus culture in petri dishes, initiating, homogenizing, maintaining, and mass-propagating the suspension culture in shake flasks and bioreactors, and finally banking the suspension production cell line are the key phases in the entire procedure of suspension culture. While a callus culture can be started using any section of a plant, it's crucial to choose the parent plant and organ type that have the appropriate quantity and quality of the bioactive component or compounds.

Plant species, development stage, location, and organ type (also known as explant) all have a significant impact on the amount and quality of the bioactive molecule or compounds of interest. In order to create a high-performing callus culture that is friable, develops and produces effectively, and is stable, growth regulators (auxins and cytokinins) that are added to the culture medium must also be considered. Secondary metabolite synthesis and callus growth and morphology are influenced by the growth regulators' type and concentration. Following the selection of callus cell lines, a suspension cell culture is produced.

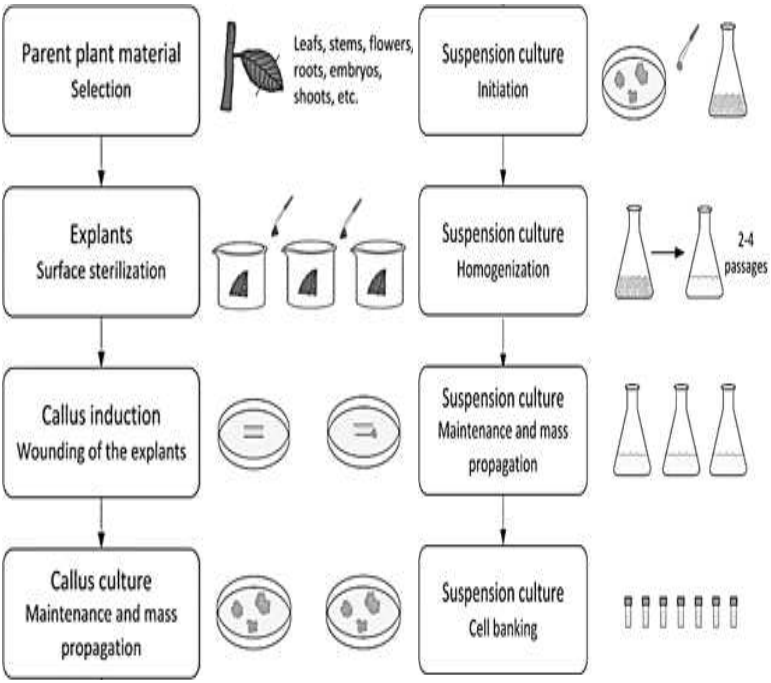

Figure 1: Standard procedure for DDC-based plant cell suspension culture (Eibl et al., 2018).

TYPES OF CELL SUSPENSION CULTURES

In a wide prospective, cell suspension cultures can be classified into two types, such as Batch cultures and Continuous cultures.

Batch cultures

Under the right circumstances, we can cultivate cells in a set quantity of culture medium using a closed system culture. To carry out this kind of cell suspension, flasks with a volume of up to 250 mL are utilized. For later flasks in suspension, the cells from the initial flask can serve as your inoculum. For each following subculture, a small aliquot is taken from the initial suspension and transferred to a new medium. The fact

that the cells grow to a point and then stop growing is a significant disadvantage of batch cultures. Both the quantity and size of cells stay unchanged during this stationary phase.

Constant cultures

In this type of cell suspension culture, we can maintain a consistent phase of cell growth. In this location, new medium is constantly brought in while the leftover nutrients and metabolic waste products are continuously removed from the medium. Therefore, by employing this technique, you can circumvent the media's detoxification and thereby get around batch cultures' disadvantage.

ADVANTAGES

- **Rapid transformation:** Transformed cell lines can be obtained and verified in a shorter timeframe than with whole plants.
- **Controlled environment**: Suspension cultures provide precise and sterile conditions, making them ideal for producing high-value recombinant proteins for clinical applications.
- **Simplified protein purification:** Depending on the expression construct employed, proteins can be readily extracted from cells or the culture medium.
- **Suitable for certain compounds:** Suspension cultures are well-suited for producing certain compounds that are not easily produced in other plant cell culture systems, like hairy roots or shooty teratomas.
- **Genetic manipulation and mutagenesis:** Suspension cultures facilitate in vitro studies for genetic manipulation, mutant initiation, and protoplast production.
- **Bioprinting and cosmetics:** They are also used in bioprinting and cosmetics industries.

LIMITATIONS

- **High scaling-up costs:** Scaling up production in large fermentors requires significant investment and specialized personnel, making it expensive.
- **Limited productivity:** Recombinant protein yields can decrease during the late stationary phase due to increased proteolytic activity, limiting overall productivity.
- **Limited cell line diversity:** The system is primarily effective with a few well-characterized cell lines, like tobacco, rice, or Arabidopsis.
- **Decreasing productivity over time:** Suspension cultures can experience a decline in productivity and slow growth over time.
- **Clumping and stickiness:** Plant cells in suspension cultures often form clumps or become sticky, requiring modifications to the culture medium or enzymatic treatments to obtain free cells.
- **Potential for contamination:** While sterile conditions are maintained, contamination risks can still be a concern, especially during large-scale production.

SUSPENSION CULTURE IN CROP IMPROVEMENT

Suspension cultures are especially useful in crop improvement for generating somaclonal variations, selecting for favourable characteristics like disease resistance or drought tolerance, and enabling genetic transformation. These cultures facilitate the mass production of secondary metabolites and can act as a basis for creating genetically modified crops with improved traits. In general, suspension culture is a significant resource for the progress of contemporary plant biotechnology and crop improvement initiatives.

BIOREACTOR

A bioreactor is a regulated space made to facilitate the development, maintenance, and control of living things (cells or microorganisms) under certain, ideal circumstances. It is frequently used to create biological products, carry out experiments, or investigate biological processes in a variety of disciplines, such as biotechnology, medicines, and research. Bioreactors maximize the growth and synthesis of desired biological substances by controlling parameters including temperature, pH, oxygen levels, and feed delivery. Recent developments in plant cell culturing in bioreactors include 3D culture methods that imitate natural conditions, microcarrier optimization for improved growth, and genetic engineering for metabolite production.

Airlift bioreactors

Airlift bioreactors, a subtype of pneumatically agitated bioreactor, utilize gas sparging and fluid circulation to establish a continuous flow of nutrients and oxygen while ensuring temperature uniformity, all without the use of mechanical agitation. Alkaloids are produced from suspension cultures of Berberis wilsoniae, with the formation of phenolic alkaloids being contingent on the level of dissolved oxygen.

Stirred-tank reactors

Continuous Stirred-tank bioreactors (CSTR) are often employed in suspension cultures, providing effective mixing via mechanical agitation, which fosters even nutrient distribution and gas exchange. They are versatile enough for both research and large-scale production, with features such as controlled temperature, pH, and dissolved oxygen levels promoting cell growth and metabolite production. Because of their scalability, stirred-tank bioreactors serve as the workhorse in industrial environments where reproducibility and yield optimization are essential.

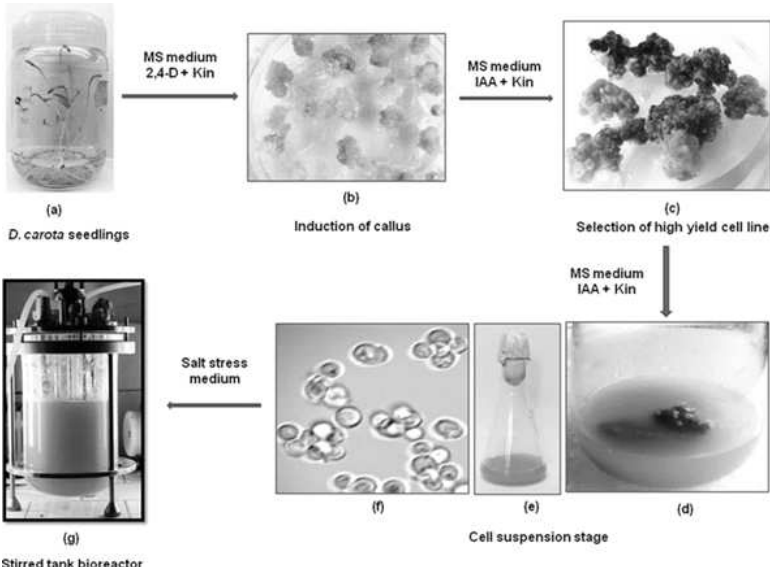

Figure 2. cell suspension culture of *D. carota*. (a) In vitro propagated, (b) callus induction on MS medium with 2,4-D and Kin, (c) anthocyanin accumulation in callus culture, (d, e) suspension culture grown in the shake flask, (f) confocal microscopy picture of anthocyanin containing cells, and (g) culture grown in the bioreactor with salt stress medium (Kirti et al., 2021).

REFERENCE

- *Eibl, R., Meier, P., Stutz, I., Schildberger, D., Hühn, T., Eibl, D. (2018). Plant cell culture technology in the cosmetics and food industries: current state and future trends.* Appl. Microbiol Biotechnol., *102(20):8661-8675. doi: 10.1007/s00253-018-9279-8. Epub 2018 Aug 11. PMID: 30099571; PMCID: PMC6153648.*
- *Indu, B.K., Balasubramanya, S., Anuradha, M., Shilpa, P. (2025). Practical Protocols of Callus and Cell Suspension Cultures. In: Anuradha, M., Balasubramanya, S. (eds)* In Vitro *Production of*

Plant Secondary Metabolites. Springer, *Singapore. https://doi.org/10.1007/978-981-97-8808-8_6*

- *Kieran, P.M., MacLoughlin, P.F., Malone, D.M. (1997). Plant cell suspension cultures: some engineering considerations. https://doi.org/10.1016/S0168-1656(97)00163-6*
- *Kirti, R. S., Gyanendra, K., Sandeep, N.M., Parvatam, G., Nandini, P. (2021). Shetty. Salt Stress-Induced Anthocyanin Biosynthesis Genes and MATE Transporter Involved in Anthocyanin Accumulation in Daucus carota Cell Culture.* ACS Omega, *6, 38, 24502–24514. https://doi.org/10.1021/acsomega.1c02941*
- *Macharoen, K., McDonald, K. A., and Nandi, S. (2020). Simplified bioreactor processes for recombinant butyrylcholinesterase production in transgenic rice cell suspension cultures.* Biochem. Eng. J., *163, 107751. doi: 10.1016/j.bej.2020.107751*
- *Mishra, N., Tripathi, M. K., Tiwari, S., Tripathi, N., Sapre, S., Ahuja, A., & Tiwari, S. (2021). Cell Suspension Culture and In Vitro Screening for Drought Tolerance in Soybean Using Poly-Ethylene Glycol.* Plants, *10(3), 517. https://doi.org/10.3390/plants10030517*
- *Moscatiello, R., Baldan, B., Navazio, L. (2013). Plant cell suspension cultures.* Methods Mol. Biol., *953:77-93. doi: 10.1007/978-1-62703-152-3_5. PMID: 23073877.*
- *Mustafa, N.R., de Winter, W., van Iren, F., Verpoorte, R. (2011). Initiation, growth and cryopreservation of plant cell suspension cultures.* Nat. Protoc., *6(6):715-42. doi: 10.1038/nprot.2010.144. Epub 2011 May 5. PMID: 21637194.*
- *Verdu´-Navarro, F., Moreno-Cid, J.A., Weiss, J., Egea-Cortines, M. (2023). The advent of plant cells in bioreactors.* Front. Plant Sci., *14:1310405. doi: 10.3389/fpls.2023.1310405*

Chapter-3

Plant Tissue Culture: Micropropagation, Organogenesis and Embryogenesis.

Sonika Kujur[1], Anjali Swain[2], Dr. R. Pushpa[3] and Dr. Samarendra Narayan Mallick[4]

[1]Regional Institute of Education, Bhubaneswar, Odisha, India.
[2]Department of Biotechnology, NIIS Institute of Information Science and Management, Bhubaneswar, Odisha, India.
[3]Plant Breeding and Genetics, Tamil Nadu Rice Research Institute, Aduthurai, Thanjavur District, Tamil Nadu
[4]Department of Botany, Ravenshaw University, Cuttack, Odisha, India.*
samarendra.mallick1@yahoo.com

ABSTRACT

Rapid clonal plant propagation under sterile circumstances is possible with the help of the potent plant tissue culture technology known as micropropagation. In micropropagation, organogenesis and somatic embryogenesis are two important developmental mechanisms. Organogenesis is the process by which explants or callus tissues develop into organs like shoots and roots. This process is usually brought about by a carefully regulated ratio of auxins and cytokinins, which are plant development regulators. On the other hand, somatic embryogenesis, which is similar to zygotic embryogenesis but does not involve fertilization, results in the creation of embryos from somatic or non-reproductive cells. For the large-scale propagation of elite or genetically modified plants, genetic conservation, and plant regeneration, both methods provide clear benefits. Enhancing the effectiveness and suitability of

micropropagation in horticulture, forestry, and agriculture requires an understanding of the physiological and molecular mechanisms that underlie these routes.

KEYWORDS: Micropropagation, organogenesis, embryogenesis.

INTRODUCTION

A contemporary method of plant tissue culture called micropropagation is utilized to quickly and extensively multiply plants in sterile lab settings. This technique makes use of plant cells' totipotency, which is the extraordinary capacity of a single cell to divide into an entire organism. It is particularly helpful for growing plants that are hard to cultivate from seeds or cuttings using traditional methods. Because of its effectiveness, speed, and capacity to create genetically identical and disease-free plants, micropropagation is used extensively in horticulture, forestry, agriculture, and plant biotechnology.

An explant, which is a tiny piece of the parent plant, like a shoot tip, leaf, or node, is carefully chosen at the start of the procedure. After being cleaned of all microorganisms with chemical disinfectants, this explant is put on a culture medium that is high in nutrients. Essential minerals, vitamins, carbohydrates, and plant growth regulators are all included in the medium, which is frequently based on the Murashige and Skoog (MS) formulation. These regulators, which are mostly auxins and cytokinins, are essential for controlling the explant's growth and determining whether it will produce callus, roots, or shoots. To guarantee ideal growing circumstances, the tissue culture is kept in a controlled environment with adjustable humidity, light, and temperature.

Organogenesis and somatic embryogenesis are the two main developmental mechanisms in micropropagation. The process of organogenesis involves the direct or indirect formation of new plant organs from the explant, such as roots and shoots.

This procedure can take place indirectly through an intermediate callus stage, or directly, where organs emerge from the explant without callus development. On the other hand, somatic embryogenesis is the process by which embryos are created from somatic (non-reproductive) cells. Although they are created asexually, these embryos resemble the normal embryonic development that takes place in seeds. This technique is particularly helpful for genetic engineering, the manufacture of synthetic seeds, and cryopreservation for long-term storage.

MICROPROPAGATION

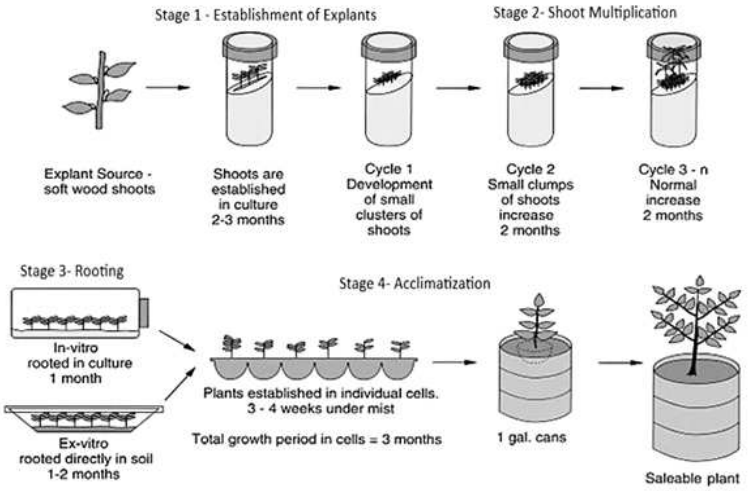

Figure 1. Stages of micropropagation.

The four primary phases of micropropagation are usually initiation, multiplication, rooting, and acclimation. The sterile explant is placed on a culture medium and starts to grow during the initiation stage. Repeated subculturing is used to induce and multiply shoots or embryoids during the multiplication stage. To encourage the production of roots, the developing shoots are moved to a medium rich in auxin during the rooting stage. Finally, in a process known as hardening, the plantlets

are progressively acclimated to outdoor circumstances, initially in a greenhouse and then in open fields or pots. Making the switch from regulated to wild settings is essential to the propagated plants' survival and well-being.

There are many benefits to micropropagation. It is perfect for commercial growing because it can produce a lot of plants in a short amount of time. Additionally, it guarantees genetic homogeneity, which is necessary to preserve desired crop qualities. Additionally, the procedure can be used to eradicate infections and create planting material free of viruses, which is crucial for crops like potatoes, sugarcane, and bananas. Because micropropagation allows for quick multiplication and preservation, it also helps conserve rare, endangered, or slow-growing organisms. Notwithstanding its many advantages, the method has certain drawbacks, such as high setup costs, the requirement for trained labor, and the possibility of contamination or somaclonal variation in the event that cultures are not adequately maintained. To sum up, micropropagation is a major development in plant biotechnology. Its rapid and efficient production of homogeneous, disease-free, high-quality plants has revolutionized the propagation of plants for commercial, conservation, and scientific uses. Micropropagation is anticipated to become even more significant in sustainable agriculture and environmental preservation as technology advances.

PROCESS

- Micropropagation relies on the totipotency of plant cells – the ability of a single cell to develop into a complete plant.
- The technique utilizes explants (small sections of plant tissue), such as shoot tips, nodes, leaves, or even single cells.
- These explants are cultured in a nutrient-rich medium containing plant growth regulators (PGRs) like auxins and cytokinins.

- Under controlled environmental conditions (light, temperature, humidity), the explant multiplies and differentiates into whole plants.

STEPS

1. Selection and Preparation of Explant

- Healthy and disease-free plant parts are selected.
- Surface sterilization using disinfectants (e.g., sodium hypochlorite) to prevent microbial contamination.

2. Initiation Phase

- Explants are placed on a nutrient medium (commonly Murashige and Skoog medium).
- Medium contains sugars, vitamins, minerals, and PGRs.
- **Objective:** Initiate cell division and callus formation or direct shoot development.

3. Multiplication Phase

- Multiple shoots or embryoids are induced by adjusting hormonal concentrations.
- Subculturing is done periodically to enhance multiplication rate.

4. Rooting Phase

- Shoots are transferred to root induction medium rich in auxins like IBA or NAA.
- Roots develop and form complete plantlets.

5. Acclimatization (Hardening)

- Plantlets are gradually introduced to external conditions.
- Initially transferred to greenhouses in controlled humidity before moving to the field.

ORGANOGENESIS

Organogenesis is a critical process in plant tissue culture

that refers to the formation of organs (shoots and roots) from undifferentiated plant cells (callus) or explants under in vitro conditions. It is a type of morphogenesis that allows the regeneration of complete plants from small tissue fragments, contributing significantly to plant biotechnology, agriculture, horticulture, and conservation efforts. This process is especially important for mass propagation of genetically uniform and disease-free plants. Organogenesis can occur either directly from explants (direct organogenesis) or indirectly via an intervening callus phase (indirect organogenesis). Hormonal balance, particularly the ratio of auxins to cytokinins, plays a key role in determining whether roots or shoots are induced.

PROCEDURE

Organogenesis in plants typically involves the following key steps:

1. Selection and Preparation of Explant

- The first step involves selecting a suitable part of the plant (explant) such as leaf, stem, root, or meristem tissue. The chosen explant must be healthy, actively growing, and genetically stable.

2. Surface Sterilization

- The explant is thoroughly sterilized using chemicals like sodium hypochlorite or ethanol to eliminate surface contaminants such as bacteria and fungi. This step ensures aseptic conditions for in vitro culture.

3. Inoculation onto Nutrient Medium

- The sterilized explant is placed onto a nutrient medium, commonly Murashige and Skoog (MS) medium, enriched with plant growth regulators (PGRs). The specific concentration of cytokinins (e.g., BAP, kinetin) and auxins (e.g., NAA, IAA) is tailored to induce shoot or root formation.

- High cytokinin:auxin ratio usually promotes shoot formation.
- High auxin:cytokinin ratio generally induces root formation.

4. Callus Formation (in Indirect Organogenesis)

- In indirect organogenesis, the explant first forms a callus (an undifferentiated mass of cells) under the influence of specific PGRs. The callus is later transferred to another medium that supports organ differentiation.

5. Organ Induction

- Under the influence of appropriate PGRs, the callus or explant starts differentiating into either shoots or roots. Multiple shoots can arise from a single explant in favorable conditions.

6. Shoot Elongation and Rooting

- Induced shoots are elongated on hormone-supplemented medium, followed by transferring to rooting medium where auxins stimulate root development.

7. Acclimatization (Hardening)

- The regenerated plantlets are moved to ex vitro conditions, where they are gradually adapted to normal environmental conditions like humidity and light. This step is vital for survival during transplantation to soil.

SOMATIC EMBRYOGENESIS

Somatic embryogenesis is a powerful and versatile technique in plant tissue culture that involves the development of embryos from somatic or non-reproductive cells, rather than from fertilized eggs (zygotes). These embryos are morphologically and developmentally similar to zygotic embryos and can give rise to entire plants. The phenomenon demonstrates the totipotent nature of plant cells, where even

fully differentiated somatic cells can be reprogrammed to initiate a complete developmental pathway. Somatic embryogenesis holds immense importance in the fields of plant biotechnology, genetic transformation, synthetic seed production, and large-scale propagation of elite cultivars. It is particularly valuable for the propagation of high-value crops, woody plants, and species that are difficult to regenerate through traditional methods.

TYPES

Somatic embryogenesis is generally categorized into two types:

1. **Direct Somatic Embryogenesis:** Embryos develop directly from explants without an intervening callus phase. This often occurs in tissues with high cellular totipotency, such as immature embryos or young leaves.
2. **Indirect Somatic Embryogenesis:** In this more common route, the explant first produces a callus—a mass of undifferentiated cells—from which somatic embryos are later induced.

PROCEDURE

The process of somatic embryogenesis generally follows these key steps:

1. **Selection and Preparation of Explant**

- The choice of explant plays a crucial role in the success of somatic embryogenesis. Explants such as immature embryos, leaf discs, hypocotyls, cotyledons, and shoot tips are commonly used. Young, actively dividing tissues are often more responsive to embryogenic induction.

2. **Sterilization of Explant**

- To maintain aseptic conditions, the explant is subjected to surface sterilization using agents like ethanol, sodium hypochlorite, or mercuric chloride, followed by

thorough rinsing with sterile distilled water.

3. Callus Induction (for Indirect Embryogenesis)

- The sterilized explant is cultured on a nutrient medium such as Murashige and Skoog (MS) medium supplemented with plant growth regulators (PGRs), particularly auxins like 2,4-Dichlorophenoxyacetic acid (2,4-D). This high-auxin medium promotes the dedifferentiation of cells and induces callus formation.

4. Embryo Induction and Development

- The callus or explant is transferred to a medium with reduced or no auxin to trigger the initiation of somatic embryos. These embryos pass through several well-defined stages resembling zygotic embryogenesis:
  A. Globular stage
  B. Heart stage
  C. Torpedo stage
  D. Cotyledonary stage

5. Embryo Maturation

- To become fully functional, somatic embryos must undergo a maturation phase. The maturation medium often includes abscisic acid (ABA) and high sucrose levels, which help in proper embryo development and desiccation tolerance.

6. Embryo Germination and Plantlet Formation

- Mature somatic embryos are transferred to germination medium containing cytokinins (e.g., BAP or kinetin) to promote shoot and root formation. These plantlets are eventually capable of autotrophic growth.

7. Acclimatization (Hardening)

- Regenerated plantlets are gradually adapted to external environmental conditions by transferring them to soil or compost in controlled environments (e.g.,

greenhouse or mist chambers). This step is essential for ensuring high survival rates during field transplantation.

APPLICATIONS OF PLANT TISSUE CULTURE

1. **Mass Propagation–** Rapid and large-scale multiplication of elite or rare plant genotypes.
2. **Genetic Engineering–** Regeneration of transgenic plants after transformation.
3. **Synthetic Seeds–** Somatic embryos can be encapsulated to produce artificial seeds.
4. **Germplasm Conservation–** Preservation of endangered species via in vitro storage.
5. **Cryopreservation–** Somatic embryos serve as excellent material for long-term cold storage.

ADVANTAGES OF PLANT TISSUE CULTURE

- **Rapid Multiplication:** Thousands of plants can be produced from a single explant in a short time.
- **Genetic Uniformity:** Produces true-to-type clones of the parent plant.
- **Disease-Free Plants:** Cultures can be cleaned of viruses and other pathogens.
- **Conservation:** Aids in preserving endangered or rare species.
- **Year-Round Production:** Not limited by seasonal changes.
- **Commercial Horticulture:** Propagation of ornamentals like orchids, lilies, and ferns.
- **Forestry:** Clonal propagation of elite tree species like eucalyptus and teak.
- **Agriculture:** Multiplication of disease-free crops like banana, potato, and sugarcane.
- **Genetic Engineering:** Used in regenerating transformed plants.

LIMITATIONS OF PLANT TISSUE CULTURE

- **Cost:** Requires expensive laboratory setup and skilled personnel.
- **Contamination Risk:** High risk of microbial contamination during culture.
- **Somaclonal Variation:** Genetic changes may occur, especially during prolonged culture.

REFERENCES

- *Desai, P., Desai, S., Rafaliya, R., Patil, G. (2022). Plant tissue culture: Somatic embryogenesis and organogenesis*. Advances in Plant Tissue Culture, *Pages 109-130. ISBN 9780323907958. https://doi.org/10.1016/B978-0-323-90795-8.00006-0*
- *Soumare, A., Diédhiou, A.G., Arora, N.K., Al-Ani, L.K.T., Ngom, M., Fall, S., Hafidi, M., Ouhdouch, Y., Kouisni, L. and Sy, M.O. (2021). Potential Role and Utilization of Plant Growth Promoting Microbes in Plant Tissue Culture*. Front. Microbiol., *12:649878. doi: 10.3389/fmicb.2021.649878*
- *Bidabadi, S. S., and Jain, S. M. (2020). Cellular, molecular, and physiological aspects of in vitro plant regeneration.* Plan. Theory, *9:702. doi: 10.3390/ plants9060702*
- *Akin-Idowu, P. E., Ibitoye, D. O., and Ademoyegun, O. T. (2009). Tissue culture as a plant production technique for horticultural crops*. Afr. J. Biotechnol., *8, 3782–3788. doi: 10.4314/ ajb.v8i16.62060*
- *Dias, J. P. T. (2019). Plant growth regulators in horticulture: practices and perspectives*. Biotecnologia Vegetal, *19, 3–14.*
- *Gupta, N., Jain, V., Rosy, M., Josepeh, Dev, S. (2020). A Review on Micropropagation Culture Method.* Asian Journal of Pharmaceutical Research and Development, *8(1):86-93. DOI: http://dx.doi.org/10.22270/ajprd.v8i1.653*
- *Bhoite, H.A. and Palshikar, G.S. (2014). Plant tissue culture: A Review.* World J. Pharma. Sci., *2:565-572*

Chapter-4

Haploid Culture Techniques : Callus Culture, Anther Culture and Ovule Culture.

Aesha Tarannum[1], Sudip Das[2], Debashree Mohanty[3] and Bikram Pradhan[4*]

[1]Centre for Biotechnology, S'O'A University, Bhubaneswar, Odisha, India.
[2]Department of Plant Physiology and Biochemistry, Bihar Agricultural University, Sabour, Bhagalpur, India.
[3]Department of Zoology, NIIS Institute of Information Science and Management, Bhubaneswar, Odisha, India.
[4]Department of Biology, Bharat Institute of Science and Technology, Bhubaneswar, Odisha, India.

ABSTRACT

In order to create haploid plants, haploid cells (which have only one set of chromosomes) are grown in vitro using haploid culture techniques. By avoiding the usual fertilization process, these methods frequently use anther or pollen culture, in which the tissues of male gametophytes are stimulated to create embryos. These techniques are important because they speed up plant breeding efforts and make genetic research easier by enabling the quick creation of homozygous lines through chromosome doubling. Additionally, haploid culture makes it possible to produce plants with unique features and examine gene expression in a reduced genomic setting.

KEYWORDS: Tissue Culture, Callus, Haploid plants, Anther culture, Ovule, Plant Biotechnology.

INTRODUCTION

In vitro plant tissue culture is a technique that allows for the growth and maintenance of plant cells, tissues, or organs under sterile conditions on a nutrient medium. It relies on the principle of totipotency, which means that a single plant cell can regenerate into an entire plant. With this method, a small piece of tissue (like a leaf, stem, or root) can be used to quickly produce genetically identical plants (clones). Tissue culture is extensively employed in agriculture, horticulture, and biotechnology for various purposes, including mass propagation, conservation of rare or endangered species, generation of disease-free plants, and genetic engineering. The procedure consists of multiple phases: choosing explants, surface sterilization, callus induction, development of shoots and roots, and plantlet acclimatization to the natural environment.

Plant tissue culture plays a major role in agriculture, providing novel methods for enhancing crops and promoting sustainability in farming practices. It is primarily used for the mass propagation of high-yielding, disease-free, and genetically uniform plants in a short time, which is particularly valuable for crops such as banana, potato, and sugarcane. It also aids in the preservation of elite and endangered plant species, contributing to the maintenance of genetic diversity. Moreover, tissue culture allows for the creation of planting material that is free from pathogens, which decreases the dependence on chemical treatments and enhances crop health. It is essential for genetic engineering, making it possible to create transgenic crops with enhanced characteristics like resistance to pests, tolerance of drought, and improved nutritional value. Furthermore, micropropagation through tissue culture supports the rapid introduction of new varieties to farmers, accelerating agricultural productivity and resilience in the face of climate change and evolving plant diseases.

CALLUS CULTURE

In the field of plant tissue culture, the method of callus culture involves generating callus (unorganized, undifferentiated cell masses) from plant explants like leaves, stems, or roots in a sterile environment. Usually, this process entails putting the explant on a nutrient medium enriched with plant growth regulators, especially auxins such as 2,4-D or NAA, and occasionally cytokinins. Calluses (Calli) provide totipotent cells that can later be prompted to develop into shoots, roots, or entire plants via somatic embryogenesis or organogenesis. Callus culture is extensively employed for plant regeneration, genetic transformation, mutagenesis, and secondary metabolite production. It is also useful for examining plant development, gene expression, and stress responses in research. This method plays a crucial role in plant biotechnology and crop improvement programs, offering a foundation for advanced applications such as synthetic seed production and genetic engineering.

HISTORY

- **Henri-Louis Duhamel du Monceau (1756):** Pioneered experiments on wound healing in plants, observing callus formation on wounded elm trees, marking the initial observation of callus in live plants.
- **Haberlandt (1902):** In 1902, Gottlieb Haberlandt was the first to establish plant callus culture. He was the first to develop the idea of cell culture in plants, establishing the basis for today's plant tissue culture methods.
- **P. White, E. Gautheret, and P. Nobécourt (1939):** Independently reported successful callus induction in vitro, using different plant species and media. White induced callus from tumor-developing tissues of Nicotiana glauca, Gautheret and Nobécourt established

continuous callus cultures of carrot using auxin hormone additions.

- **Nobécourt (1937-1939):** Established the first callus culture capable of continuous growth on a semisolid agar medium, using a *Daucus carota* tap root explant.
- **J. Van Overbeck, M.E. Conklin, and A.F. Blakeslee (1941):** Reported the importance of coconut milk in callus culture, providing essential nutrients for plant cell growth.
- **S.M. Caplin and F.C. Steward (1948):** First successfully grew differentiated, non-cambial cells from *Daucus carota* using coconut milk in the medium, later demonstrating cell division induction with synthetic auxin.
- **1960s onwards:** Research focused on understanding cell behavior, metabolism, and morphogenesis in callus cultures, leading to applications in plant pathogen eradication, germplasm storage, and clonal propagation.

CALLUS TYPES

Based on parameters such as texture, color, and cellular characteristics, plant callus can be classified into several types, which often indicate the tissue's physiological state and regenerative potential. The common types of calluses are as follow:

- **Friable:** Marked by cells that are loosely grouped together, can easily break apart, and often have a white or creamy yellow appearance.
- **Compact:** Features tightly packed cells, with a green and robust appearance.
- **Regenerating**: Calli capable of developing into complete plants via organogenesis (formation of shoots and roots) or embryogenesis (development of somatic embryos). They can be further divided into shoot calli, root calli, or embryogenic calli.

- Non-Regenerating: Calli utilized for cell suspension cultures and metabolite production that do not generate organs.

PROTOCOL FOR CALLUS CULTURE

Callus culture, a widely used method in plant tissue culture, involves inducing and sustaining undifferentiated cell masses (callus) from plant explants in a sterile environment. Below is a general protocol for callus culture:

Explant Selection and Preparation:

- Select an explant (a segment of plant tissue) that has a good chance of forming callus.
- Surface-sterilize the explant to eliminate any microbial contamination. Common methods involve the use of ethanol, bleach, or mercury bichloride.
- Prepare the explant by cutting it into smaller segments and handling it correctly to prevent contamination.

Medium Preparation:

- Prepare a sterile nutrient medium that supplies the essential nutrients and minerals for callus growth.
- Well-known media comprise Murashige and Skoog (MS) medium, White's medium, and woody plant medium.
- To induce callus formation, incorporate plant growth regulators into the medium, including auxins (such as 2,4-D or NAA) and cytokinins (like BA or kinetin).

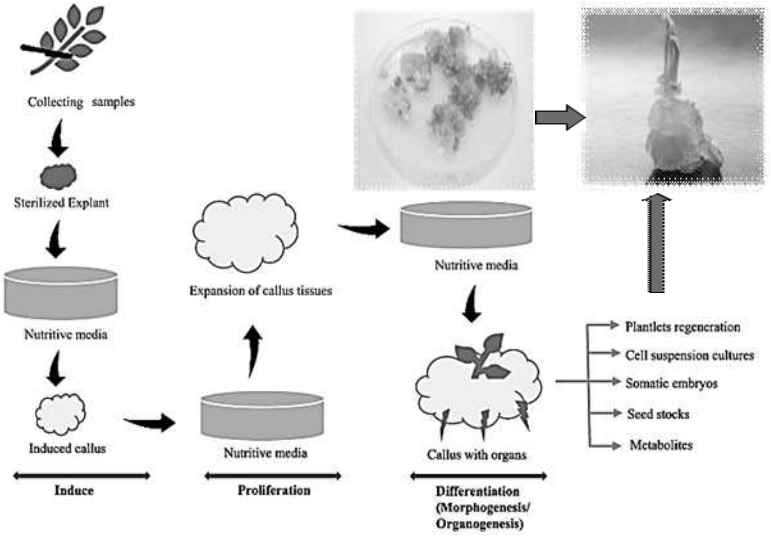

Figure 1. The picture represents various stages of callus culture.

Inoculation and Incubation:

- Disinfect the explant and place it on the prepared medium.
- Place the culture in a controlled environment for incubation, ensuring proper conditions for temperature (typically 25-28°C), light (16 hours of light and 8 hours of darkness), and humidity.

Callus Proliferation and Maintenance:

- Observe the growth of the callus and modify conditions as necessary.
- To ensure healthy growth and avoid nutrient depletion, periodically subculture the callus.

APPLICATION OF CALLUS CULTURE IN AGRICULTURE

Callus culture is significantly useful in agriculture, especially for plant breeding and enhancing crops. A major application

of this technique is in plant regeneration via tissue culture methods, which allows for the swift propagation of genetically uniform plants that exhibit beneficial characteristics like enhanced yield, disease resistance, or drought tolerance. It serves as a crucial step in genetic transformation, allowing for the introduction of foreign genes into callus tissue, which can then be regenerated into complete transgenic plants. Moreover, callus culture fosters somaclonal variation, which provides a genetic diversity source that can be utilized to create new cultivars. In crop biotechnology, it facilitates the mass production of secondary metabolites, thereby increasing the value of medicinal and aromatic plants.

LIMITATIONS OF CALLUS CULTURE

- **Genetic Instability:** Somaclonal variation can arise from callus cultures, leading to undesirable genetic mutations that might impact the uniformity and stability of regenerated plants.
- **Low Regeneration Efficiency:** Certain plant species or callus types do not regenerate efficiently into whole plants; some may remain undifferentiated or necrotic.
- **High Cost and Labor Intensive:** Ensuring sterility, preparing culture media, and ongoing subculturing demand specialized tools, considerable time investment, and trained individuals.
- **Hormone Sensitivity:** The effectiveness of callus induction and regeneration is largely determined by the exact equilibrium of plant growth regulators, a factor that can differ greatly among species and even among explants.
- **Tissue Browning and Necrosis:** The oxidation of phenolic compounds in certain explants can result in browning, which may cause callus death and diminish viability.

ANDROGENESIS

In plant tissue culture, androgenesis refers to the process whereby male reproductive cells (microspores or immature anther cells) develop into haploid plants without the need for fertilization. This method is essentially for anther and microspore culture, and it is commonly employed in genetic research and plant breeding. In androgenesis, the microspore is diverted from its typical route of developing into sperm cells and instead follows a developmental trajectory that results in the formation of a complete plant. Haploid plants produced in this way have only one set of chromosomes, which makes them very useful for generating homozygous lines after chromosome doubling. This speeds up the breeding process and aids in creating new crop varieties with desirable characteristics like disease resistance, stress tolerance, and enhanced productivity.

POLLEN CULTURE

Pollen culture, or androgenesis, is a method of plant tissue culture that generates haploid plants from immature pollen grains (microspores) in sterile conditions. The success of anther culture can be affected by factors like the plant species, genotype, and so on. Haploid plants, which contain only one set of chromosomes, make it possible for breeders to quickly generate homozygous lines after chromosome doubling. This is why anther culture is commonly employed in plant breeding programs. This method is essential for plant breeding as it allows for the creation of homozygous lines through haploid chromosome doubling, which greatly shortens the time needed to attain genetic uniformity in comparison with conventional methods. Pollen culture is crucial for producing doubled haploids (DHs), which are essential in the creation of stable, high-yield, disease resistance, and stress tolerance crop varieties.

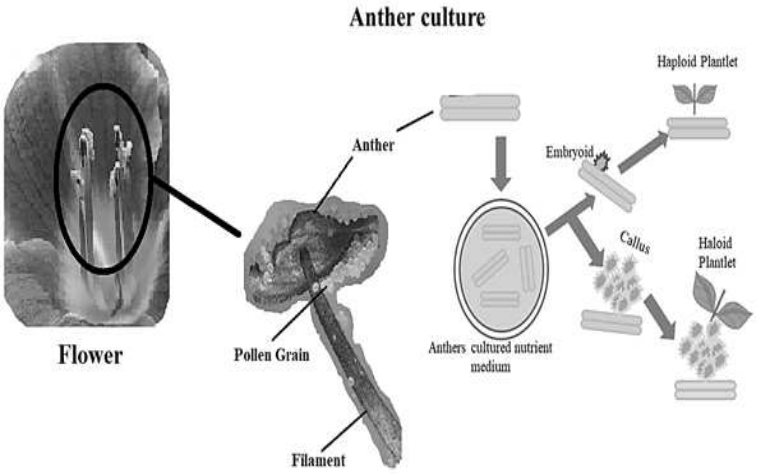

Figure 2. Haploid plant production by using anther culture technique.

Additionally, it enables the examination of gene expression and mutation at the haploid level, which can be beneficial for functional genomics. Widely applied in crops such as rice, wheat, and barley, pollen culture contributes to accelerated crop improvement programs, making it a powerful tool in modern agriculture and plant biotechnology.

GYNOGENESIS

In plants, gynogenesis is a type of asexual reproduction in which an embryo forms from the female gamete (egg cell) without any genetic input from the male parent. Embryo development can be triggered without fertilization by using irradiated or genetically inactive pollen, which can artificially induce this process, often through in vitro techniques. The plants that result are haploid, containing solely the maternal chromosome set, which can later be doubled to generate homozygous diploids. Gynogenesis is especially beneficial in plant breeding programs, as it allows for the quick generation

of pure lines and accelerates the creation of new cultivars. It has been effectively used in various crop species, such as onion, wheat, and sugar beet.

OVULE CULTURE

Ovule culture, a technique in plant tissue culture, involves growing ovules under sterile in vitro conditions to aid embryo development, particularly when normal fertilization or seed development is hindered. This approach is especially useful for surmounting obstacles in interspecific and intergeneric hybridization, where fertilization can take place but embryo loss occurs prior to seed development. Ovule culture is a crucial component of embryo rescue methods, as it enables the preservation of embryos and their growth into healthy plants by cultivating ovules at suitable points in their development. It also finds application in haploid production through the culturing of unfertilized ovules, as well as in genetic research related to early embryo development and seed physiology. Ovule culture plays a significant role in plant breeding and genetic enhancement programs by facilitating the recovery of hybrids that would otherwise be lost, thereby broadening the genetic diversity available for crop improvement.

APPLICATION OF HAPLOID CULTURE

Haploid culture is of great importance for agriculture, especially in the areas of plant breeding and crop enhancement. Breeders can rapidly create homozygous diploid lines through chromosome doubling by producing haploid plants that contain only one set of chromosomes. This fosters the creation of genetically uniform and stable pure lines, which is crucial for hybrid seed production and trait fixation. Haploid culture is particularly effective for reducing breeding cycles, increasing selection efficiency, and recognizing advantageous genetic characteristics like disease resistance, drought tolerance, and yield improvement. It has been extensively used in crops such

as rice, wheat, maize, and barley, aiding in the creation of enhanced varieties that fulfil the requirements of contemporary agriculture.

REFERENCE

- *Ali, J., Nicolas, K.L.C., Akther, S., Torabi, A., Ebadi, A.A., Marfori-Nazarea, C.M., Mahender, A. (2021). Improved Anther Culture Media for Enhanced Callus Formation and Plant Regeneration in Rice (*Oryza sativa *L.).* Plants (Basel), *10(5):839. doi: 10.3390/plants10050839. PMID: 33921954; PMCID: PMC8143452.*
- *Aysar, M., Mohammed Salim, A. (2020). CALLUS INDUCTION AND DIFFERENTIATION (Review Article).* The future of agriculture, *5-9. 10.37229/fsa.fja.2020.07.02.*
- *Ikeuchi, M., Sugimoto, K., Iwase, A. (2013). Plant callus: mechanisms of induction and repression.* Plant Cell, *25(9):3159-73. doi: 10.1105/tpc.113.116053. Epub 2013 Sep 27. PMID: 24076977; PMCID: PMC3809525.*
- *Jin, S. B., Kim, M. J., Choi, C. W., Park, S. M., & Yun, S. H. (2022). Anther Culture-Derived Haploids of* Citrus aurantium *L. (Sour Orange) and Genetic Verification of Haploid-Derived Regenerated Plants.* Plants, 11*(22), 3022. https://doi.org/10.3390/plants11223022*
- *Keller, E.R.J., Korzun, L. (1996). Ovary and ovule culture for haploid production. In: Jain, S.M., Sopory, S.K., Veilleux, R.E. (eds)* In Vitro *Haploid Production in Higher Plants.* Current Plant Science and Biotechnology in Agriculture, *vol 23. Springer, Dordrecht. https://doi.org/10.1007/978-94-017-1860-8_13*
- *Nesi, B., Ghiselli, L., Gori, M., Natale, R., Tomiozzo, R., Mansuino, A., & Biricolti, S. (2023). "In Vitro" Ovule Culture to Improve Genetic Variability in* Hydrangea macrophylla. Horticulturae, 9*(9), 1028. https://doi.org/10.3390/horticulturae9091028*

- *Song, J., Datla, R., Zou, J., Xiang, D. (2024). Haploid induction: an overview of parental factor manipulation during seed formation.* Front. Plant Sci., *15:1439350. doi: 10.3389/fpls.2024.1439350*
- *Upadhyay, R. (2022). Anther Culture For Haploid Plant Production. 10.1016/B978-0-323-90795-8.00004-7. https://www.researchgate.net/publication/362964620_Anther_Culture_For_Haploid_Plant_Production*

Chapter-5

Synthetic Seeds and its Significance

Nafisa Ekka[1], Sudip Das[2], Pragyan Paramita Panda[3] and Dr. Samarendra Narayan Mallick[4*]

[1]Regional Institute of Education, Bhubaneswar, Odisha, India.
[2]Department of Plant Physiology and Biochemistry, Bihar Agricultural University, Sabour, Bhagalpur, India.
[3]Department of Botany, NIIS Institute of Information Science and Management, Bhubaneswar, Odisha, India.
[4]Department of Botany, Ravenshaw University, Cuttack, Odisha.*

ABSTRACT

A ripe ovule that contains an embryo that may regenerate is termed a seed, and it is the link that binds the kingdom of plants together from generation to generation. Artificially encapsulated somatic embryos, shoot buds, cell aggregates, or any other tissue that may be used for seeding and that has the capacity to develop into a plant in vitro or ex vitro, as well as the ability to do so after storage, are referred to as synthetic seeds. Promising plant genotypes can be mass-propagated using the artificial seed production approach, which is also an alternative propagation option for many commercially significant crops. Synthetic seed technology was created for a variety of economically significant plant species, including cereals, spices, fruit crops, ornamental plants, orchids, medicinal plants, wood-bearing forest trees, industrially important commodities, vegetable crops, fodder legumes, and fruit crops. The elite agricultural and endangered medicinal plant species, which are hard to restore using traditional methods and natural seeds, can be multiplied and conserved

with the help of this technology.

KEYWORDS: Synthetic seeds, Crop improvement, Plant breeding, Biotechnology, Hybrid.

INTRODUCTION

In nature, the main means of plant propagation is usually seeds. Certain plants can be propagated vegetatively, but traditional techniques are costly, time-consuming, and unable to generate seedlings on a wider scale. The creation of artificial seeds, or Syn-seeds, may be greatly aided by synthetic seed technology. Plant micropropagules, such as somatic embryos, shoot buds or tips, calli, nodal segments, embryogenic masses, protocorms, and protocorm-like entities, are encapsulated with particular coating materials to create artificial or synthetic seeds. The encapsulated plant tissues are protected and nourished by the outer covering matrix. Because it improves capsule formation and gives alginate beads enough hardness to survive mechanical damage to propagules, calcium alginate gel is the most popular protective coating available. In order to promote the normal development of plant propagules, which results in Synthetic Seed germination and the generation of healthy plants, nutrients, growth regulators, antibiotics, and other adjuvants are added to the coated matrix.

SYNTHETIC SEED

Synthetic seed-based technologies that encapsulate somatic embryos, shoot tissues, or axillary buds in an appropriate matrix have a significant impact on plant propagation by facilitating the exchange of germplasm, enhancing genetic preservation, and facilitating effective genetic modification. This opens up the possibility of planting new seeds and achieving shared objectives. One of the most promising plant biotechnology tools is synthetic seed technology, which can be used in horticultural

and agricultural development studies now and in the future with specific three procedures. A synthetic encapsulation procedure that permits plant components, such as shoot tips, cell aggregates, or somatic embryos, to demonstrate their capacity to mature into a whole plant even after prolonged preservation in in vitro or ex vitro settings is commonly referred to as synthetic seed. For many commercially significant agricultural and horticultural species, synthetic seed manufacturing technology is regarded as an efficient and successful alternative propagation strategy. One particularly effective method for mass-propagating elite plant species with significant commercial potential is synthetic seed. Essential agricultural crops can now be improved thanks to advancements in biotechnology research over the past few decades. Since the production of synthetic seeds offers numerous commercial advantages for the development of a variety of agricultural crops, it has expanded the potential of the in vitro plant propagation approach. Because it offers substantial advantages for the large-scale creation of unique (elite) plant species, it is one of the most crucial instruments for scientists and plant tissue culture breeders.

NATURAL SEED VS SYNTHETIC SEED

Natural seeds, resulting from the fertilization of ovules, are produced through sexual reproduction in plants. They consist of an embryo, stored nutrients, and a protective seed coat, and they germinate when environmental conditions are suitable. On the other hand, synthetic seeds consist of somatic embryos or alternative plant tissues that are encased artificially and have the potential to grow into a complete plant. These are developed in labs through plant tissue culture methods and are mainly used for the mass propagation of plants exhibiting desirable characteristics. Natural seeds, which are found in nature and exhibit genetic variation, differ from synthetic seeds that are typically uniform in genetics and facilitate the swift multiplication of high-quality plants without disease.

TYPES

Various plant propagules are enveloped in coating materials that function as synthetic endosperms, supplying nutrients to embryos while also serving a protective purpose. Depending on the various methods employed to produce them in accordance based on the requirements, synthetic seeds can be broadly categorized into dehydrated and hydrated seeds.

- **Desiccated man-made seedlings**: Somatic embryos get encapsulated in polyoxyethylene and are subsequently dried under controlled conditions. Desiccation can occur at a slow or fast pace, depending on the circumstances. Immediate desiccation involves opening a sealed Petri dish containing syn-seeds and leaving it open overnight for rapid drying, whereas slow desiccation of the encapsulating seeds in a room with decreasing humidity takes one to two weeks. For plant species whose somatic embryos are resistant to desiccation, synthetic desiccated seeds can be produced.

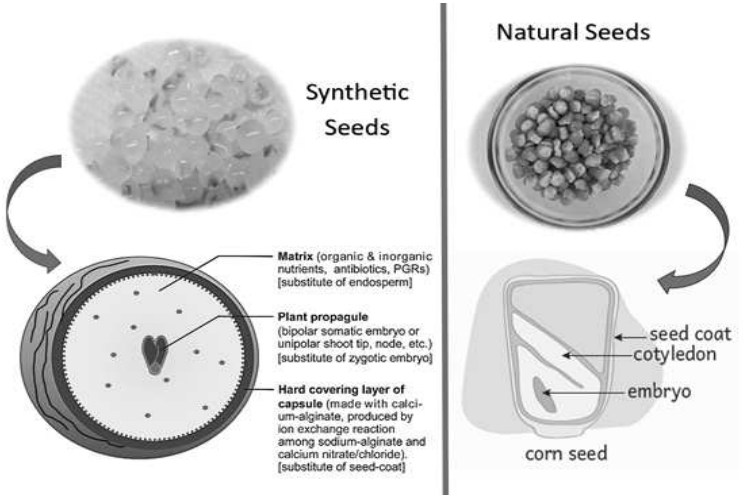

Figure 1. Internal structure of synthetic and natural (corn) seeds.

- **Hydrated synthetic seed**: Hydrated artificial seeds consist of somatic embryos or suitable plant tissues encased in hydrogel. Many materials, such as potassium alginate, agar, gelrite, and sodium pectate, have been examined. Nonetheless, calcium alginate has demonstrated the greatest effectiveness as a coating material for wet synthetic seeds.

KEY MILESTONES

Scientist	Events
Haberlandt (1902)	Tissue culture technique
Karl Ereky (1919)	Term "Biotechnology"
Jost (1941)	Term "Genetic Engineering"
Stewart (1958)	Somatic embryogenesis in carrot
Murashige (1978)	Term " Synthetic Seed"
Drew (1979)	Somatic embryos as seed delivery system
P.S.Rao (1980)	Synthetic seeds developed at BARC
Lawrence (1981)	Encapsulation technique
Redenbaugh (1986)	Hydrogel Encapsulation technique
Fujii (1989)	Grow plants from synthetic seeds

PROTOCOL FOR SYNTHETIC SEED PRODUCTION

Standard Protocol for Artificial Seed Production

1. Selection of Plant Material

- Choose a plant species suitable for in vitro propagation.
- Collect explants such as leaves, shoot tips, or immature embryos.

2. Induction of Somatic Embryogenesis

- Sterilize explants.
- Culture on a suitable induction medium (e.g., MS medium supplemented with auxins like 2,4-D or NAA).
- Incubate under dark/light conditions depending on species for 1–3 weeks.

3. Development and Maturation of Somatic Embryos

- Transfer induced callus to maturation medium (MS medium with reduced auxin, often with ABA or activated charcoal).
- Incubate for 2–4 weeks until fully formed bipolar somatic embryos develop.

4. Encapsulation

- Prepare **encapsulation matrix** (commonly sodium alginate 2–5% w/v).
- Prepare **complexing solution** (usually calcium chloride 50–100 mM).
- Mix somatic embryos with sodium alginate solution.
- Drop the mixture into CaCl, solution to form **beads** via ion exchange gelation.
- Allow beads to harden for 20–30 minutes.
- Rinse beads in sterile water to remove excess CaCl, .

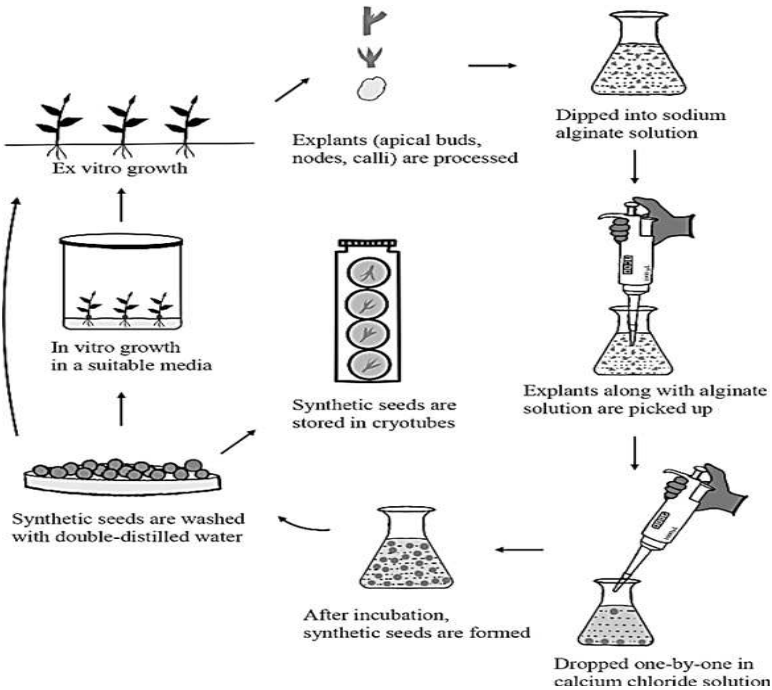

Figure 2. A schematic diagram representing different stages of synthetic seed production under in vitro condition by using plant tissue culture technology (**Subrahmanyeswari et al., 2024**).

5. Optional Additives in the Beads

- Nutrients (e.g., MS salts, sucrose)
- Growth regulators (e.g., BAP, GA*f*)
- Antimicrobials or fungicides
- Activated charcoal

6. Storage (Optional)

- Artificial seeds can be stored at 4°C for short-term storage (1–2 months).
- Desiccation-tolerant formulations can allow longer storage.

7. Germination and Plantlet Regeneration

- Sow encapsulated beads on sterile germination medium (MS or ½ MS medium).
- Maintain in growth chamber at ~25°C with a 16/8 h light/dark cycle.
- Somatic embryos germinate and develop into plantlets in 2–4 weeks.

8. Acclimatization

- Transfer regenerated plantlets to soil or potting mix under high humidity.
- Gradually expose to normal environmental conditions.

ADVANTAGES

With the synthetic seed technology, it will be possible to propagate medicinal plants on a large scale by generating uniform, disease-free specimens in great numbers within a short timeframe and at minimal expense. The synthetic seed technology can tackle various problems, including low seedling

viability, poor seed germination rates, slow seed multiplication, and seasonal dependence on traditional methods of vegetation reproduction. By developing a convenient and efficient method for the storage and transport of plant material, synthesized seed technology can be integrated into the germplasm of medicinal plant storage. Additionally, it is possible to create synthetic seeds that prevent genetic erosion and reduce the metabolic content of medicinal plants by copying or closely mimicking the genotype and phenotype of explants

LIMITATIONS

However, the tech-free seed technology has its shortcomings as well, such as inconsistent conversion and germination rates. These inconsistencies can contribute to a lack of reliability in synthetic seed production. Various factors affect the germination and conversion rates of synthetic seeds, including the type of explants, the encapsulating agent used, the matrix composition, storage conditions, germination conditions, among others. Therefore, it is necessary to optimise the protocols for each plant or species and various types of synthetic seeds to achieve better results.

SYNTHETIC SEED FOR CROP IMPROVEMENT

By facilitating the large-scale reproduction of plants that are disease-free and genetically superior, synthetic seeds contribute greatly to crop enhancement. These seeds are created by encasing somatic embryos or other plant tissues in a gel-like medium, which facilitates easy handling, storage, and transport. Synthetic seeds play a crucial role in crop enhancement initiatives by safeguarding superior genotypes and swiftly propagating plants that exhibit beneficial characteristics like high yield, pest resistance, or drought tolerance. They are particularly useful for crops that are hard to propagate using traditional seeds or have low seed viability. Synthetic seeds promote greater efficiency in breeding, accelerate the spread

of improved varieties, and boost agricultural productivity by guaranteeing quality and uniformity.

ROLE OF SYNTHETIC SEED IN AGRICULTURE

In contemporary agriculture, synthetic seeds are significant because they provide an effective means for propagating and conserving high-quality plant varieties. They are especially beneficial for producing elite or genetically modified plants on a large scale, which are not easy to propagate using traditional seeds. Synthetic seeds guarantee crop uniformity, lower the chances of disease spread, and facilitate cultivation throughout the year, independent of seasonal constraints. Additionally, they make it easier to store and transport valuable plant material while ensuring that its viability is not significantly compromised. Moreover, synthetic seeds aid conservation endeavors by safeguarding rare, at-risk, or slow-growing plant species. Synthetic seeds enhance crop productivity, reduce reliance on traditional seed systems, and support advanced breeding and biotechnology programs, thereby contributing to sustainable agriculture.

REFERENCE

- *Chandra, K., Pandey, A., Kumar, P. (2018). Synthetic seed–Future pro-spects in crop improvement.* Int J. Agric. Innov. Res., *6(1):120-25.*
- *Liu, C., He, Z., Zhang, Y., Hu, F., Li, M., Liu, Q., et al. (2022). Synthetic apomixis enables stable transgenerational transmission of heterotic phe-notypes in hybrid rice.* Plant Communications, *4(2):1-9. https://doi.org/10.1016/j.xplc.2022.100470*
- *Mangena, P. (2021). Synthetic seeds and their role in agriculture: status and progress in sub-Saharan Africa.* Plant Sci. Today, *8(3):482-90. https://horizonepublishing.com/journals/index.php/PST/article/view/1116*
- *Özbay, S., Isak, M. A., & ªimºek, Ö. (2024). Synthetic Seed Pro-*

duction and Cryopreservation for Myrtle (Myrtus communis L.) Genotypes. Çukurova Tarým Ve Gýda Bilimleri Dergisi, *39(1), 126-136. https://dergipark.org.tr/en/pub/cutarim/issue/85563/1420329*

- *Rihan, H.Z., Kareem, F., El-Mahrouk, M.E., Fuller, M.P. (2017). Artificial seeds (principle, aspects and applications).* Agronomy, *7(4):71. https://www.mdpi.com/2073-4395/7/4/71*
- *Rojas-Vásquez, R., Zuñiga-Umaña, J.M., Abdelnour-Esquivel, A., Hernández-Soto, A., Gatica-Arias, A. (2022). Development of synthetic seeds in Arabica coffee embryos under aseptic and non-aseptic conditions.* Vegetos., *35(3):839-49. https://doi.org/10.1007/s42535-022-00364-9*
- *Sahu, S. and Singh, B. A. (2024). The Journey of Artificial Seeds in Modern Agriculture.* Vigyan Varta, *5(7): 71-75.*
- *Sahu, S., Monalisa, S.P., Jigile, S., Kar, S., Parida, S.S., Swain, S.K. (2024). Exploring the potential of synthetic seeds: Influence of explant, encapsulating agent and matrix, advantages and challenges.* Plant Science Today, *(Early Access). https://doi.org/10.14719/pst.3931*
- *Subrahmanyeswari, T., Mahanta, M., Bandyopadhyay, S. (2024). Production, Storage, and Regeneration of Synthetic Seeds from Selected Medicinal Plants with Antiallergic Property. In: Gantait, S., Majumder, J., Sharangi, A.B. (eds) Biotechnology of Medicinal Plants with Antiallergy Properties.* Springer, *Singapore. https://doi.org/10.1007/978-981-97-1467-4_19*

Chapter-6

Hybridization Techniques: Embryo Rescue, Somatic Hybridization and Cybrids

Manaswini Jena[1], Dr. R. Pushpa[2], Soumyajit Nayak[3] and Dr. Somali Das[3*]

[1]Bagchi Sri Shankara Cancer Centre and Research Institute, Bhubaneswar, Odisha, India.
[2]Plant Breeding and Genetics, Tamil Nadu Rice Research Institute, Aduthurai, Thanjavur District, Tamil Nadu, India.
[3]Department of Biotechnology, NIIS Institute of Information Science and Management, Bhubaneswar, Odisha, India.
sumisomali22@gmail.com

ABSTRACT

Embryo rescue is a plant tissue culture method for preserving and cultivating immature or weak embryos that would otherwise fail to develop, particularly in cases of interspecific or intergeneric hybridizations where normal seed development is hindered. This approach is essential for plant breeding programs and assists in overcoming post-zygotic barriers. By fusing two different protoplasts (cell without walls) from separate species or genera, somatic hybrids are created that combine their nuclear and cytoplasmic genomes. Using this technique, it is possible to create new plant varieties that possess characteristics of both parent species, even when sexual reproduction cannot occur. Cybrids, also known as cytoplasmic hybrids, are created by retaining the nucleus from one parent while using cytoplasm (which includes organelles such as mitochondria or chloroplasts) from both parents, often via selective fusion or enucleation. Cybrids are particularly useful for incorporating cytoplasmic characteristics like disease

resistance or male sterility into preferred cultivars.

KEYWORDS: Plant Biotechnology, Seedless, Embryo rescue, Somatic Hybrids, Cybrids.

INTRODUCTION

A key component of fast breeding is the rapid advancement of generations. Breeding programs can be reduced by cutting the generation period, which enables the creation of novel varieties to meet market needs, urgent climate change challenges, and the need for more sustainable agriculture. Changing agronomic practices that are known to impact generation time in a variety of crops is one way to shorten generation time. For instance, because it is not necessary to obtain physiologically ripe seeds for the following generations, embryo rescue can be utilized to drastically reduce the generation time in intraspecific crosses in addition to its long-standing use in distant crosses to produce hybrids.

Additionally, endosperm and other tissues must be removed from embryos, perhaps in order to avoid infection. If not, any pathogen source could infect other tissues, initially halting embryonic growth and ultimately causing their viability to end. Research on crossbreeding grapes with varying numbers of chromosomes also makes use of the embryo rescue procedure. It is simpler to produce viable hybrid genotypes when diploid grape varietals are utilized as the female parents. Additionally, tetraploid types are said to have reduced ovule fertility than diploid varieties. The success rate varies in different weeks based on the parent combination, despite the fact that various studies have demonstrated the importance of the sampling time for embryo recovery.

EMBRYO RESCUE

Embryo rescue is a method that uses in vivo plant growing

settings to transform hybrid embryos (intraspecific and interspecific hybrids that cannot survive in normal conditions) into viable plants. This approach requires special attention to hygiene. The process entails cultivating mature or mature-lethal embryos in vitro using a certain nutritional culture medium. Climate chambers with varying food types and climatic conditions are necessary, particularly depending on the species.

Successful crossings have been made possible via embryo rescue, which has also been used to produce haploids and double haploids, alter ploidy levels for monosomic and disomic insertion, and engineer chromosomes. Furthermore, uncommon plant propagation and breeding cycle reduction are possible through embryo rescue. It has also been employed numerous times to investigate different stages of embryonic development, particularly in mutants that are embryo-lethal. Directly putting underdeveloped embryos in the culture medium is the most often utilized embryo rescue technique. Sometimes the effective development of young embryos from the zygote stage to maturity is made possible by the in vitro culture of ovaries, ovaries, or placentas.

The success rate of embryo rescue can also be impacted by plant growth regulators, various culture conditions, and the embryo development medium. The results show that the chosen species or varieties themselves are the most important of these, and that they can be successful during the sampling period, even though many other aspects are mentioned as being important in the success rate of the experiments that were carried out. Furthermore, it has been demonstrated in recent years that incorporating several Vitis species into these investigations can significantly raise the success rate.

HISTORY

- In the 18th century, Charles Bonnet (1720–1793) made groundbreaking strides in embryo rescue (ER) by delicately excising mature embryos from common beans

and buckwheat and then adeptly transferring them into soil for growth.

- During 1904, E. Hanning successfully cultured mature embryos of various plant species on a mineral salt medium with sugar, marking the beginning of in vitro embryo culture in plants.
- In 1925, Laibach introduced embryo culture as a tool for overcoming interspecific hybridization barriers in plants.
- Brinster optimized basic parameters like pH, osmolality, and energy substrates for embryo culture, leading to advancements in media development in 1960s.

APPLICATION OF EMBRYO RESCUE

1. Overcoming pre-zygotic and post-zygotic Barriers in Hybridization

The incompatibility reaction in the pre-zygotic type frequently leads to the lack of pollen germination, pollen tube growth, or pollen tube penetration into the ovule. This can happen at different levels in different tissues, including the ovary, style, or stigma. Cut-style or graft-on-style methods, the utilization of pollen mixtures from many species, placenta pollination, and in vitro ovule pollination have all been employed to get beyond pre-zygotic obstacles. The kind of genetic barrier (i.e., incompatibility) that inhibits fertilization determines the methods to be used. If the incompatibility is post-zygotic, it could be necessary to cultivate immature embryos. The adoption of the best ER approaches is made more difficult by interspecific and intergeneric hybridizations, which block a number of post-zygotic barriers, the mechanisms of which are yet unclear.

2. Abiotic and Biotic Stress Resistance Crops

Embryo deliverance (ER) is a group of procedures used to deliver mongrel embryos produced from interspecific and

intergeneric crosses that are not suitable for in vivo survival or conventional factory parentage procedures, as well as immature/mature-murderous embryos. Immature or deadly embryos are extracted, and they are then cultivated in vitro on a certain nutrient culture medium. When trying to recover immature embryos, it is important to take into account the experimental differences between dicots and monocots. To differentiate between what occurs in the normal embryonic development of dicot and monocot embryos, special attention is paid to nutrient species-specific conditions and the evaluation of growth condition metrics. Many genes governing resilience to biotic and abiotic stressors were lost during the domestication of shops. The initial claims of the domestication process were the revision of key characteristics, such as coetaneous fruit growth, absence of antinutrients, frustrations and waxes, factory armature, seed dormancy, fruit and seed size, conciseness of cognizance, and absence of seed disbandment. As a result, factories are now totally dependent on mortal care for survival, a phenomenon called as the domestication pattern. Unfortunately, it was common to ignore the stress-resistant traits of wild parent species.

3. Enhancement of economically significant crops

Crop enhancement has long been a top priority in order to feed the growing number of people who are dying. The blossoming features in stores form the basis of all breeding strategies since they induce inheritable rearrangement. Conventional parentage styles can be used to improve flowers genetically because they can set seeds following either natural or artificial pollination. Regretfully, every crop eventually reaches a point where it can no longer be improved upon due to its exhausted inheritable pool. In order to find seeker genes with desired features, the breeders are searching for crop wild cousins. Unusual walls are typically produced when cultivated and wild cousins cross-fertilize. Congruity and contradiction,

however, cause unsynchronized floral organ production and disrupt postfertilization seed setting when it comes to incompatibility. Even so, environmental circumstances and inheritable incompatibilities might cause the embryo to revert following successful fertilization. At the molecular level, postfertilization recap factors aid in the feasible seed product and inhibit embryo growth. The process known as "embryo deliverance" (ER) involves gutting interspecific hybridization embryos in vitro and dressing them on medium to prevent revocation. One of the earliest in vitro factory parenting techniques is ER. Crop genotype, culture conditions, factory growth regulators, fruit age, and the most important phases of the rescued embryos are some of the many variables that affect how effective the ER fashion is. The torpedo stage embryo has the highest success rate when compared to other embryo stages. For field and horticultural crops, the ER fashion was a boon for interspecific and intergeneric mongrels. unseasonable germination, malnutrition, cytological changes during embryogenesis, endosperm balance number, and polar capitals activation are several triggers of interspecific hybridization failure that can be resolved using this fashion. Seed physiology, viability, and dormancy, as well as ploidy and colourful chromosome elimination exploration, all benefit from ER. As a result, this system has been veritably effective for the interspecific mongrel development in a colourful vegetable, fruits, and cosmetic and field crops. The ER fashion's facilitates farmers, factory breeders, and the seed assiduity for global food and nutrition security. This chapter provides streamlined and detailed information about ER ways, influences, and operations in marketable crops.

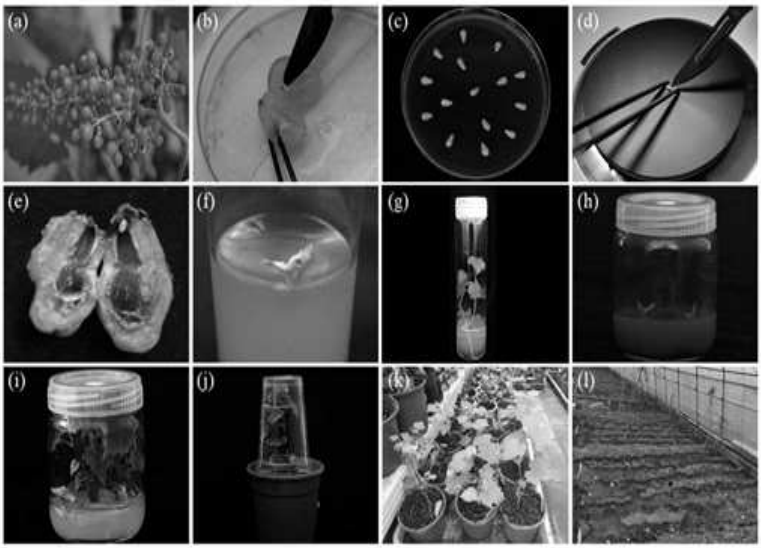

Figure 1. The embryo rescue process: (a) hybrid fruits; (b) ovule collection; (c) ovule inoculation; (d) embryo excision; (e) excised embryo; (f) immature embryo germination; (g) plantlet from germinated embryo; (h) subculture; (i) plantlet from secondary culture; (j) seedlings harden; (k) seedling in greenhouse; (l) seedlings in field **(Chu et al., 2023)**.

SOMATIC HYBRIDIZATION

The fashion of emulsion of insulated protoplasts from physical cells and rejuvenescence of mongrel shops from the emulsion products is called physical hybridization which fully bypasses the coitus and allows combining the genomes of two desirable parents, irrespective of their taxonomic relationship. The mongrel cells that are formed from the emulsion of two unconnected protoplasts combines a set of three genomes from the parents, viz. nuclear genome, mitochondrial genome, and plastid genome. operations of physical hybridization in crop enhancement are constantly evolving. still, it must be appreciated that genomic incompatibility following protoplast

emulsion continues to be a serious debit in physical hybridization. Factory physical hybridization through protoplast emulsion is an important tool in factory enhancement, which allows experimenters to combine physical cells (whole or partial) from different cultivars, species or rubrics performing in new inheritable combinations including symmetric mongrels, asymmetric mongrels or cybrids. physical hybridization has a characteristic eventuality to combine both nuclear and cytoplasmic genes contemporaneously unlike sexual hybridization or inheritable engineering ways. This fashion can grease parentage and gene transfer, bypassing problems which are associated with conventional sexual crossing, including sexual incompatibility, polyembryony, manly or womanish sterility. Listing (1960) published his colonist work on factory protoplast insulation. Still, the first physical mongrel product was reported by Carlson et al. (1972) in the rubric tobacco through the fashion of cell emulsion. This has now been extended to a large number of rubrics to produce symmetric physical mongrels (with complete nuclear genomes of both the parents), asymmetric mongrels (nuclear genome from the patron parent into the genome of the philanthropist parent), and cybrids (nuclear genome of a parent with mitochondrial genome of the other parent). Since, also hundreds of reports have been published during the once three decades which extend the procedures to fresh factory rubrics and estimate the application eventuality of physical mongrels in numerous crops species like rice, rapeseed, tomato, potato and citrus.

PROCEDURE

The essential way followed in the fashion of physical hybridization are:

I. Insulation of protoplasts-

Protoplast insulation has been reported from mesophyll cells of in vivo and in vitro grown shops, sterile seedlings,

embryogenic and non-embryogenic suspense societies, cotyledons, hypocotyls, and manly and womanish gametes. The youthful leaves from in vitro grown sterile shoot societies are the extensively used towel to insulate protoplasts. The mesophyll cells in the leaves are approximately arranged hence, the enzymes have an easy access to the cell wall. Insulation of protoplasts requires at least two enzymes like a pectinase enzyme to dissolve the middle lamina that binds the conterminous cells together and a cellulose enzyme to digest the cell walls and release the protoplasts.

II. Emulsion of protoplasts-

Protoplasts that have been freshly isolated will fuse when they are brought into close contact and held together for several minutes. The techniques most commonly employed to fuse plant protoplasts are chemical fusion using PEG (polyethylene glycol) and electric stimulation. PEG is widely recognized as a plant protoplast fusogen due to its ability to induce a high frequency of heterokaryon formation while maintaining low toxicity to plant cells. The original PEG method and high Ca2+/high pH method are commonly combined and used. Heterokaryons refer to the fused protoplasts containing two nuclei from different parents, while homokaryons are those with nuclei from the same parent. During the culture process, the nuclei of the heterokaryons fuse together to form a hybrid cell.

III. Culture of protoplasts to raise entire factory-

The protoplasts produce a well-defined cell wall and losses its spherical shape property within 24 hours when cultured under optimal conditions. In general, a proper somatic cell wall is necessary for the cell's functioning. The duration necessary for the first cell division in protoplast cultures is influenced by various factors, including the species and genotype, the origin of the protoplasts, the isolation method employed, their viability, the formulation of the culture media, and the environmental conditions of cultivation.

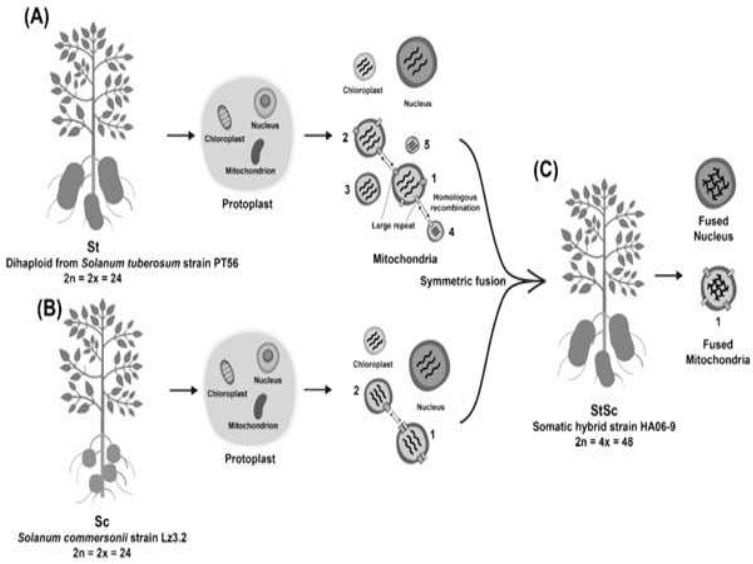

Figure 2. Development of somatic hybrid of *Solanum* species by protoplast fusion techniques **(Cho et al., 2022)**.

IV. Selection of hybrid cells-

A number of approaches have been used to select or increase the population of hybrid cells. Among these, biochemical mutants and resistance to antibiotics and herbicides are employed quite often. Up to this point, the biochemical mutants lack chlorophyll or nitrate reductase and are albino mutants that have been extensively utilized

APPLICATION

- Generating new interspecific and intergeneric hybrids: By enabling the merging of characteristics from species or varieties that are not closely related, somatic hybridization provides a solution to the constraints of conventional sexual hybridization.
- **genes for disease resistance:** Through somatic hybridization, it is possible to transfer genes that confer

resistance to diseases from one plant to another, resulting in hybrids that are resistant to a range of pathogens such as viruses, bacteria, and fungi.

- **Enhancing tolerance to environmental stress:** Through somatic hybridization, plants can be produced that have improved resistance to abiotic stresses such as heat, cold, drought, and salinity.
- **Transfer of cytoplasmic characteristics:** Somatic hybridization enables the introduction of cytoplasmic traits such as cytoplasmic male sterility (CMS), antibiotic resistance, and herbicide resistance, which are not usually transferred via sexual hybridization.
- **Cybrids development:** Cybrids can be generated through the donor-recipient method or cytoplast–protoplast fusion, and they can also arise spontaneously from intraspecific, interspecific, or symmetric hybridization across different genera.

CYBRIDS

Typically, in sexual hybridization, only the female parent contributes the plastid and mitochondrial genomes, while in somatic hybridization, the extranuclear genomes of both parents are combined. Consequently, the latter approach of the crossing of plants offers an unusual opportunity to investigate the interactions of cytoplasmic organelles. New combinations of nuclear/plastid/mitochondrial genomes are generated in plants via interparental mitochondrial genome recombination and the separate assortment of chloroplasts and mitochondria following cell fusion. A plant is called a cybrid if its nuclear genome mainly comes from one of the fusion partners, while it contains some organelle genomes from the other fusion partner.

HISTORY

- Klercker was the first to use a mechanical method to isolate protoplasts in 1892.

- In 1960, Cocking was the first to report the isolation of protoplasts from tomato root tips using concentrated cellulase solutions derived from fungi.
- PEG was first proposed for protoplast fusion by Kao and Michayluk in 1974.
- In 1979, Gleba fused tobacco protoplasts to create a cybrid.

ADVANTAGES

- Two distinct parental genomes that cannot engage in sexual reproduction (either asexual or sterile) are recombined.
- Cybrids surpass obstacles of sexual incompatibility.
- Cybrids utilized in research of cytoplasmic genes and their functions - experiments in plant breeding.
- Utilized for transferring characteristics of antibiotic resistance (tobacco)
- Cybrids serve the purpose of transferring herbicide resistance (brassica).
- It is also applied in mitochondrial research.

LIMITATIONS

- Only a few genera exhibit biparental inheritance of cytoplasm during sexual reproduction.
- It is often difficult, or even impossible, to regenerate a plant from a protoplast.
- Unstable behaviour of genes transferred in somatic hybrids.
- Recovering controlled asymmetric hybrids as a result of processes such as cell fusion, nuclear fusion, and recombination.

REFERENCE

- *Backiyarani, S., Uma, S., Saranya, S., Durai, P., Perianayagaraj, S.E., Selvaraj, V., Saraswathi, M.S., Karthic, R., Kalpana, S.*

(2021). Multiple shoot induction in zygotic embryos: A strategy for acceleration of banana breeding. Plant Cell Tissue Organ Cult. *147, 339–350.*

- *Begna, T. (2020). Review on Somatic Hybridization and its Role in Crop Improvement.* International Journal of Research Studies in Agricultural Sciences (IJRSAS), *6(6):25-37. DOI: http://dx.doi.org/10.20431/2454-6224.0606004*
- *Cho, KS., Lee, HO., Lee, SC.* et al. *(2022). Mitochondrial genome recombination in somatic hybrids of* Solanum commersonii *and* S. tuberosum. Sci Rep., *12, 8659. https://doi.org/10.1038/s41598-022-12661-z*
- *Chu, Y., Li, M., Li, R., Zhang, K., Qiu, P., Yuan, X., Han, Y., Liu, X., Xu, Y., Liu, G. (2023). Embryo Rescue Breeding of New Cold-Resistant, Seedless Grapes.* Horticulturae, *9, 992. https://doi.org/10.3390/horticulturae9090992*
- *Doyðacý, Y., Boztepe, Ö., Kandilli, G.G. et al.(2024). Embryo recovery(rescue) studies in different Vitis species.* BMC Plant Biol., *24, 822. https://doi.org/10.1186/s12870-024-05539-x*
- *Seeja, G. and Sreekumar, S. (2020). A review on cybrids: An approach for plant improvement.* Crop Res. *55: 48-56. DOI: 10.31830/2454-1761.2020.011.*
- *Holmes, M. (2018). Somatic Hybridization: The Rise and Fall of a Mid-Twentieth-Century Biotechnology.* Historical Studies in the Natural Sciences, *48(1):1–23. https://doi.org/10.1525/hsns.2018.48.1.1*
- *Kang, M., Lee, K., Finley, T., Chappell, H., Veena, V., Wang, K. (2022). An improved* Agrobacterium*-mediated transformation and genome-editing method for maize inbred B104 using a ternary vector system and immature embryos.* Front. Plant Sci. 13, *860971.*
- Krishna, R., Khandagale, K., Benke, A.P., Soumia, P.S., Manjunathagowda, D.C., Ansari, W.A., Mokat, D.N., Gawande, S.J., Ade, A.B., Singh, M. (2022). Embryo

rescue: A potential tool for improvement of economically important crops. In *Advances in Plant Tissue Culture, Current Developments and Future Trends*; Chapter 11; Rai, A.C., Kumar, A., Modi, A., Singh, M., Eds.; Academic Press: Cambridge, MA, USA, pp. 259–282. ISBN 9780323907958.

- *Prajwalitha, K., Bahadur, V., & Mishra, S. (2025). Somatic Hybrids and Cybrids: Innovations in Vegetable Improvement.* International Journal of Plant & Soil Science, *37(1), 331–346. https://doi.org/10.9734/ijpss/2025/v37i15275*
- *Singh, A., Mishra, N., Kumar, S., and Pandey, S. (2015). "Application of Somatic Hybridization for the Improvement of Horticultural Crops".* Horticultural Biotechnology Research, *vol. 1, pp. 39-45, doi:10.19071/jhcbt.2015.v1.2897.*

Chapter-7

Modern Plant Breeding Technology: Somaclonal Variation, rDNA Technology and Cryopreservation

Dr. Sudip Das[1], Dr. R. Pushpa[2] and Dr. G. Narayana Swamy[3].

[1]Department of Plant Physiology and Biochemistry, Bihar Agricultural University, Sabour, Bhagalpur, India.
[2]Plant Breeding and Genetics, Tamil Nadu Rice Research Institute, Aduthurai, Thanjavur District, Tamil Nadu, India.
[3]Horticulture Agriculture college, Pulivendula, ANGRAU, Andhra Pradesh, India.

ABSTRACT

Utilizing cutting-edge instruments and techniques, modern plant breeding technology increases crop resilience, quality, and output. Plant genomes can be precisely altered through the use of molecular markers, genomic selection, genetic engineering, and genome editing methods like CRISPR/Cas9. Improved crop varieties can be developed more quickly thanks to these technologies, which make it easier for breeders to find and incorporate advantageous genes. Furthermore, the quick reproduction of elite lines and the preservation of genetic resources are facilitated by tissue culture and micropropagation methods. When combined, contemporary plant breeding technologies provide long-term answers to the problems of global food security and climate change adaptation.

KEYWORDS: Somaclonal variation, hybrid, crops, cryopreservation, rDNA, Plant Biotechnology.

INTRODUCTION

Numerous horticultural species can now be regenerated in vitro thanks to developments in tissue culture techniques, and a variety of crops can now be multiplied on a commercial scale using micropropagation processes. A high level of genetic homogeneity among the regenerated plants is necessary for clonal proliferation and the maintenance of elite genotypes that were chosen for their exceptional traits. However, genetic variability (somaclonal variations) may be produced by plant tissue culture due to gene mutation or modifications in epigenetic markers. A disadvantage of both germplasm preservation and in vitro cloning is the presence of minor somaclonal variation. As a result, ensuring the genetic homogeneity of plants grown in vitro at an early stage is crucial. The genetic integrity of the progenies produced in vitro has been determined using a variety of techniques, including morpho-physiological, biochemical, cytological, and DNA-based molecular marker approaches. Somaclonal variation can pose a considerable obstacle in any micropropagation endeavor where the production of true-to-type plant material is highly sought after. However, in horticultural crops that are either hard to breed or have a limited genetic foundation, somaclonal variation has given breeders a new and alternative option for obtaining genetic variability reasonably quickly and without complex technology.

Somaclonal variation in plant biotechnology denotes the genetic alterations that arise in plants produced from tissue culture, a method often employed for micropropagation and genetic modification. These variations result from the artificial conditions of in vitro culture, including hormonal imbalances, extended culture durations, and the dedifferentiation and redifferentiation of plant cells. Somaclonal variation, though it can be seen as a barrier to the creation of genetically uniform clones, is also an effective resource for enhancing crops. It offers a natural way to achieve genetic diversity without resorting to

traditional breeding methods or genetic engineering. Somaclonal variation also refers to the genetic variation observed among plants that have been produced through plant tissue culture techniques, such as callus culture, somatic embryogenesis, or organogenesis. This variation can occur spontaneously as a result of cellular stress, mutations, chromosomal rearrangements, or epigenetic changes during the in vitro culture process. Somaclonal variation can be both beneficial and detrimental: while it may lead to undesirable traits that affect plant quality or uniformity, it also serves as a valuable source of novel genetic diversity for plant breeding and crop improvement. Screening somaclonal variants for favorable characteristics like higher yield, stress tolerance, or disease resistance can be performed by biotechnologists, making it a valuable approach for creating improved plant varieties.

Despite its potential benefits, somaclonal variation can have several negative impacts, particularly in commercial plant propagation where genetic uniformity is essential. Unintended genetic or epigenetic changes can lead to undesirable traits such as reduced vigor, abnormal growth patterns, lower yield, or altered flowering and fruiting characteristics. These variations can compromise the quality and consistency of plant products, making them unsuitable for large-scale agriculture or horticulture. In some cases, somaclonal variation may also result in increased susceptibility to diseases or environmental stress. As a result, careful screening and quality control are necessary in tissue culture programs to detect and eliminate undesirable variants, ensuring the production of genetically stable and high-quality plant material.

HISTORY

Somaclonal variation has its origins in the early advancements of plant tissue culture in the middle of the 20th century. Even though the regeneration of plants from cultured cells was first shown to be possible in the 1950s, it wasn't until

the late 1970s and early 1980s that researchers started to systematically observe and record genetic variability among regenerated plants. Larkin and Scowcroft introduced the term "somaclonal variation" in 1981 to refer to the heritable variation observed in plants regenerated from somatic (non-reproductive) cells in culture. This finding called into question the prior belief that tissue culture would invariably yield genetically identical clones, paving the way for advancements in plant biotechnology and crop enhancement.

TYPES OF VARIATION

Somaclonal variation is categorized into two primary types: genetic variations and epigenetic variations. Genetic variations refer to alterations in the DNA sequence, including point mutations, chromosomal rearrangements, deletions, insertions, or polyploidy. These changes are stable and can be inherited, frequently resulting in lasting modifications to plant traits. In contrast, DNA sequences are not changed by epigenetic variations; rather, they influence gene expression via mechanisms like histone modification or DNA methylation. These alterations can be reversed and are not always transmitted to the next generation. Both forms of somaclonal variation can affect plant morphology, physiology, and development, making them significant factors in plant tissue culture and crop enhancement initiatives.

FACTORS INDUCING VARIATION

Numerous stressors have been identified as the causes of tissue culture mutations, such as wounding, exposure to sterilants during sterilization, incomplete tissue (protoplasts are an extreme example), media component imbalances like high concentrations of plant growth regulators (auxin and cytokinins), sugar from the nutrient medium replacing photosynthesis in the leaves, lighting conditions, and the disturbed relationship between high humidity and transpiration.

A significant portion of the variability observed in micropropagated plants could be caused by or connected to oxidative stress damage to plant tissues that occurs during in vitro culture. Oxidative stress leads to an increase in pro-oxidants or reactive oxygen species (ROS), including superoxide, hydrogen peroxide, hydroxyl, peroxyl, and alkoxyl radicals. These ROS may contribute to altered hyper- and hypo-methylation of DNA, changes in chromosome number ranging from polyploidy to aneuploidy, chromosome strand breakage, rearrangements of chromosomes, and deletions and substitutions of DNA bases, which in turn can result in mutations in plant cells *in vitro*.

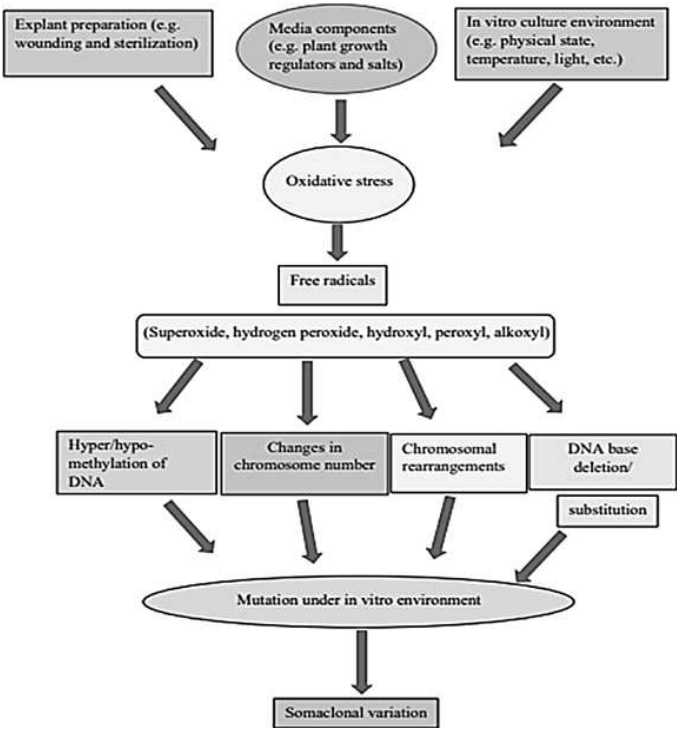

Figure 1. Graphical presentation of various stages in Somaclonal variation.

a. Variation from mother explants

Somaclonal variation may arise from somatic mutations present in the tissues of the donor plant. Somatic embryos obtained from the first regeneration round can be subjected to a further in vitro regeneration round to check for pre-existing somaclonal variation. Tissues exhibiting pre-existing variation are expected to produce greater variability in the first somaclonal generation compared to the second. Consequently, the variation in the second round can be reduced or stabilized.

b. Phytohormones in culture medium

Polyploidy may be induced by auxins and cytokinins being present in unbalanced concentrations, while cells exhibit normal ploidy when growth regulators are present at low levels or not at all. Moreover, a rapid and disorganized growth process can lead to somaclonal variation. Suboptimal and supraoptimal levels of growth regulators, especially synthetic compounds, have been linked to somaclonal variation. When auxins are added to cultures of unorganised calli or cell suspensions, the rate of DNA methylation increases, thereby enhancing genetic variation. Likewise, in strawberry callus cultures, the synthetic auxin 2,4-Dichlorophenoxyacetic acid (2,4-D) is frequently linked to genetic irregularities like polyploidy and enhanced DNA synthesis, potentially leading to endoreduplication.

c. Frequency of culture cycles

With a greater number and longer duration of subcultures, somaclonal variation occurs more often, especially in callus cultures and cell suspensions. In addition, the swift amplification of tissues or cultures developed over time may impact genetic stability, resulting in somaclonal variation. A statistical model has been suggested for estimating the theoretical mutation rate, mainly based on the number of multiplication cycles. Nevertheless, the model's applicability is restricted because of the intricacy of biological systems.

ROLE IN CROP IMPROVEMENT

Somaclonal variation denotes the genetic diversity seen in plants that have been regenerated from somatic cells cultured in vitro. This variation is caused by mutations, chromosomal rearrangements, or epigenetic changes that happen during the tissue culture process. Somaclonal variation is a useful resource in crop enhancement for creating new characteristics, including disease resistance, stress tolerance, and yield improvement. Somaclonal variation can introduce new traits without crossbreeding, unlike traditional breeding that relies on existing genetic diversity. This renders it particularly beneficial for crops with narrow genetic bases or in situations where conventional breeding poses difficulties. Somaclonal variation plays a role in creating enhanced crop varieties that possess desirable agronomic characteristics.

Somaclonal variation is particularly important in banana improvement, as bananas are mostly propagated vegetatively and have limited genetic diversity. Through in vitro tissue culture techniques like micropropagation, somaclonal variation can lead to the emergence of new traits, offering a valuable source of genetic variability. This variation has been effectively used to develop banana plants with improved resistance to major diseases such as Fusarium wilt (Panama disease) and Black Sigatoka, as well as enhanced tolerance to abiotic stresses like drought and salinity. In addition, somaclonal variation can result in improved yield, fruit quality, and shelf life. Since conventional breeding in banana is difficult due to sterility and parthenocarpy in many commercial varieties, somaclonal variation provides an efficient alternative for generating and selecting beneficial traits, thereby supporting the development of more resilient and productive banana cultivars.

Through tissue culture techniques, such as callus induction and plant regeneration, variations arise at the genetic and epigenetic levels, which can lead to desirable traits in rice plants.

These include improved tolerance to abiotic stresses like drought, salinity, and cold, as well as enhanced resistance to pests and diseases such as bacterial blight and blast. In addition, somaclonal variation has been used to develop rice lines with better yield, grain quality, and maturity traits. Since rice has a relatively narrow genetic base, somaclonal variation offers an alternative approach to conventional breeding by creating new traits that may not be found in natural populations.

RECOMBINANT DNA (RDNA) TECHNOLOGY

Recombinant DNA (rDNA) technology represents a potent method in genetic engineering, which entails merging DNA from diverse origins to produce novel genetic combinations. With this method, scientists can isolate, alter, and add specific genes to an organism's genome, making it possible for desired traits to manifest. In the agricultural sector, rDNA technology has been employed to create genetically modified (GM) crops that feature enhanced traits like resistance to pests, tolerance for herbicides, improved nutritional value, and extended shelf life. In the field of medicine, it allows for the manufacturing of crucial drugs such as insulin, human growth hormone, and vaccines. The procedure usually employs restriction enzymes for DNA cutting, ligases for the joining of DNA fragments, and vectors like plasmids to insert the recombinant DNA into host cells. By offering accurate and effective methods for altering genetic material for research, industrial, agricultural, and medical purposes, rDNA technology has transformed the field of biotechnology.

IMPORTANT MILESTONES

- **1960s:** Werner Arber, Hamilton Smith, and Daniel Nathans identified restriction enzymes, which cut DNA at specific sequences, paving the way for manipulating DNA.
- **Early 1970s:** Paul Berg demonstrated the feasibility of

splicing and recombining genetic material, creating the first recombinant DNA molecules in vitro.

- **1973:** Stanley Cohen and Herbert Boyer, building on Berg's work, successfully inserted recombined genes into bacterial cells, demonstrating that engineered DNA could be replicated and expressed in foreign cells.
- **1974:** Stanford University applied for a patent on recombinant DNA technology, marking a significant step in its commercialization.
- **1970s-1980s:** Restriction enzymes and reverse transcriptase were discovered, enabling scientists to generate recombinant DNA molecules and leading to advancements in medicine, agriculture, and industry.
- **1982:** The first genetically modified crop plant, an antibiotic-resistant tobacco plant, was produced.
- **1986:** The first field trials of genetically engineered plants occurred in France and the US, with tobacco plants engineered for herbicide resistance.
- **1990s:** The "Gene Revolution" era began, with rDNA technology leading to improved crop yield, economic benefits for farmers and consumers, and reduced environmental impacts of agriculture.

PRINCIPLE OF RDNA TECHNOLOGY

Recombinant DNA (rDNA) technology operates on the principle of cutting and splicing together DNA from various sources in order to forge a new genetic combination that possesses specified characteristics. The initial step involves identifying and isolating a gene of interest from the donor organism. The gene is then placed into a suitable vector (i.e. plasmid) which serves as a carrier to deliver the gene into a host organism. To cut the donor DNA and vector at predetermined locations, restriction enzymes are utilized, while DNA ligase is employed to link the resulting fragments. The recombinant DNA is introduced into the host cell, where it

can replicate and express the desired gene. This enables the creation of genetically modified organisms (GMOs) that possess particular advantageous characteristics. rDNA technology is based on precision, which allows scientists to manipulate genetic material at the molecular level for use in agriculture, medicine, and industry.

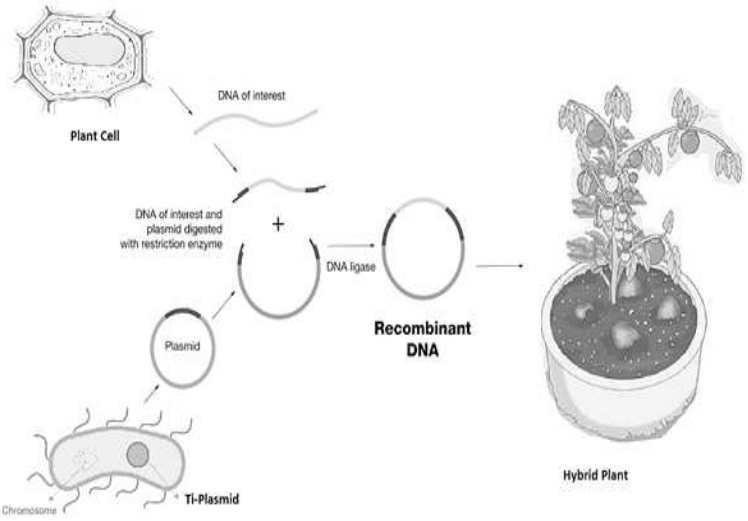

Figure 2. Production of hybrid plants by using rDNA technology.

APPLICATION

Recombinant DNA (rDNA) technology has transformed plant biotechnology, providing many new methods for crop development and enhancement. A major application is the development of genetically modified (GM) plants with improved characteristics, such as pest, disease, and herbicide resistance, which results in lower pesticide application and better yields. It is also utilized to create crops with enhanced nutritional value, like Golden Rice, which is fortified with vitamin A to address malnutrition. Through rDNA technology,

it is possible to add genes that confer drought and salinity tolerance, aiding plants in enduring difficult environmental conditions. Moreover, it aids in the creation of plant-produced pharmaceutical compounds (molecular farming) and fosters research by generating model organisms with reporter or marker genes.

CRYOPRESERVATION

The process of cryopreservation serves to maintain biological specimens like cells, tissues, embryos, or seeds at extremely low temperatures—usually in liquid nitrogen at -196°C. At such temperatures, all metabolic and biochemical processes cease, enabling genetic material to be stored over the long term with minimal loss of viability or function. In the field of plant conservation, cryopreservation is commonly employed, particularly for safeguarding the genetic resources of species that are rare, threatened, or of commercial significance. In agriculture, it is essential for the upkeep of germplasm collections, the provision of a consistent supply of superior plant varieties, and the facilitation of breeding initiatives. The procedure requires meticulous preparation, such as employing cryoprotectants to avert ice crystal formation that can harm cells. In summary, cryopreservation is an effective means in biotechnology and conservation for the secure long-term storage and retrieval of important genetic resources.

It involves freezing plant materials such as seeds, embryos, meristems, or somatic cells at ultra-low temperatures, typically in liquid nitrogen at -196°C, where all cellular activities are suspended. This method ensures the preservation of plant germplasm without genetic alteration over extended periods. In plant biotechnology, cryopreservation supports the conservation of endangered species, the maintenance of elite breeding lines, and the secure storage of genetically modified or tissue-cultured plants. It is especially useful for species with recalcitrant seeds or those that do not produce viable seeds. By

enabling the safe and cost-effective storage of valuable plant material, cryopreservation plays a crucial role in biodiversity conservation, crop improvement programs, and sustainable agricultural development.

APPLICATION

Cryopreservation guarantees a steady provision of elite and genetically diverse germplasm for breeding programs by preserving the viability and genetic stability of stored material. It is especially useful for conserving rare, endangered, or clonally propagated species that cannot be preserved through standard methods. Tissues that have been cryopreserved can be regenerated into complete plants for the purpose of reintroducing beneficial characteristics, including disease resistance, stress tolerance, or enhanced yield. Moreover, it aids biotechnological methods such as genetic transformation and somaclonal variation by offering a reliable backup of important lines. Therefore, for genetic conservation and breeding purposes, as well as for the sustainable development of enhanced crop varieties, cryopreservation is a vital resource.

REFERENCE

- *Dorani, E., Dehghanian, Z., Gougerdchi, V., Hamedpour-Darabi, M. (2024). Application of Somaclonal Variation in Crop Improvements. In: Kumar, N. (eds) Plant Mutagenesis. Sustainable Landscape Planning and Natural Resources Management. Springer, Cham. https://doi.org/10.1007/978-3-031-50729-8_8*
- *Krishna, H., Alizadeh, M., Singh, D., et al. (2016). Somaclonal variations and their applications in horticultural crops improvement.* 3 Biotech, *6:54. DOI 10.1007/s13205-016-0389-7*
- *Ferreira, M.d.S., Rocha, A.d.J., Nascimento, F.d.S., Oliveira, W.D.d.S., Soares, J.M.d.S., Rebouças, T.A., Morais Lino, L.S., Haddad, F., Ferreira, C.F., Santos-Serejo, J.A.d., et al. (2023). The Role of Somaclonal Variation in Plant Genetic Improvement:*

A *Systematic Review.* Agronomy, *13*, *730*. *https:// doi.org/ 10.3390/agronomy13030730*

- *Duta-Cornescu, G., Constantin, N., Pojoga, D.-M., Nicuta, D., Simon-Gruita, A. (2023). Somaclonal Variation–Advantage or Disadvantage in Micropropagation of the Medicinal Plants.* Int. J. Mol. Sci., *24*, *838*. *https://doi.org/10.3390/ijms24010838*
- *Efferth, T. (2019). Biotechnology applications of plant callus cultures.* Engineering, *5*, *50–59*.
- *Alfalahi, A.O., Hussein, Z.T., Sadder, A.K.M.T., Qasem, J.R., Al-Khayri, J.M., Jain, S.M., Almehemdi, A.F. (2022). Epigenetic variation as a new plant breeding tool: A review. J. King Saud Univ. Sci., 34, 102302.*

Chapter-8

Genetic Transfer Methods: Gene Gun, PEG techniques and *Agrobacterium* mediated.

Meniswani Rout[1*], Dr. R. Pushpa[2], Sweta Singh and Bikram Pradhan[3]

[1]Department of Biotechnology, College of Basic Science & Humanities, Orissa University of Agriculture and Technology, Bhubaneswar, Odisha, India.*
[2]Plant Breeding and Genetics, Tamil Nadu Rice Research Institute, Aduthurai, Thanjavur District, Tamil Nadu, India.
[3]Department of Biology, Bharat Institute of Science and Technology, Bhubaneswar, Odisha, India.

ABSTRACT

The ability to transfer genes from one organism to another has been made possible in recent years by the gene transfer technique. These days, gene technology is developing at an incredible rate and is used in a wide range of fields, from food to health, from plants to animals. The ongoing development of gene transfer techniques altered the course of crop modification and led to important breakthroughs in agricultural output, crop protection, and crop enhancement. The process of finding and introducing new genes to already-existing elite cultivars is known as genetic engineering, and it has completely changed the crop improvement pathway. Numerous techniques have been devised to introduce the gene into plant cells, and ongoing endeavors have been undertaken to enhance its effectiveness. There are advantages and disadvantages to both direct and indirect gene transfer strategies. Constant work was done to remove obstacles and create a simple, prestigious, and eco-friendly gene transfer

technique. Agrobacterium-mediated gene transfer and the gene gun transformation approach have shown promising results in recent years. The Agrobacterium gene transfer technique is particularly popular for introducing foreign genes into a wide variety of plant species. The methodology in question, as well as the advantages and disadvantages of the different approaches, were briefly covered together.

KEYWORDS: Gene Gun, Microinjection, PEG, *Agrobacterium*, Plant Biotechnology

INTRODUCTION

In genetic engineering, molecular biology, and biotechnology, gene transfer techniques are essential instruments. In order to investigate gene function, create genetically modified species, and further gene therapy, they make it possible to introduce foreign genetic material into cells. Gene transfer is the process of changing a cell or organism's genetic composition by transferring genetic material, such as DNA or RNA. This method is essential in domains such as gene therapy, biotechnology, and genetic engineering. It allows researchers to create genetically modified organisms (GMOs), conduct research on gene functions, and treat a variety of diseases. The development of novel cultivars that will increase production stability and quality, protect the environment, provide consumers with nutritional benefits, and provide new medications and vaccines are the main goals of plant biotechnology.

TYPES OF GENE TRANSFER

Gene Transfer occurs in two primary ways-

1. **Vertical Gene Transfer: -** The natural transfer of genetic material from parent to child during reproduction is known as vertical gene transfer. In both sexual and

asexual reproduction, it is the typical mode of inheritance.

2. **Horizontal Gene Transfer: -** The transfer of genetic material between species that are not related by parent-offspring is known as horizontal gene transfer, or HGT. Particularly prevalent in bacteria, HGT is important for the development of novel characteristics and the spread of antibiotic resistance.

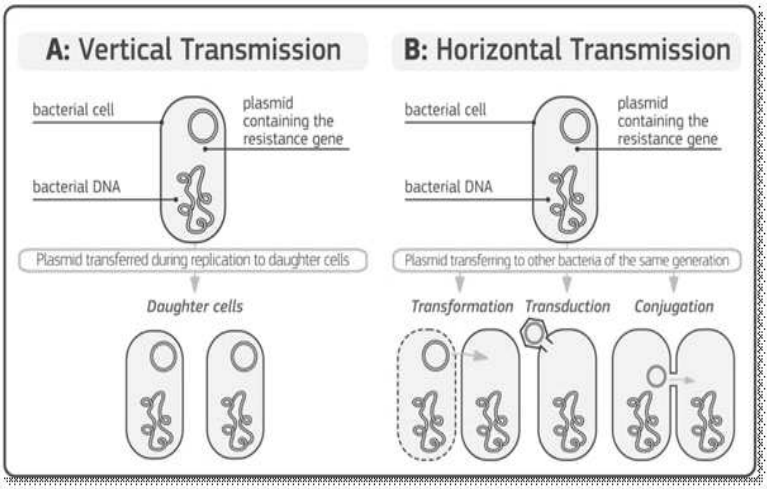

Figure 1. Types of gene transfer.

Plant biotechnology's use of this gene transfer system and its tactics has produced plants that are resistant to pests and diseases, delayed the senescence of leaves, modified the composition of fatty acids, produced flowers with a variety of colors and patterns, developed storage traits, and produced plants that are resistant to stress.

IMPORTANCE OF GENE TRANSFER

A. Provide resistance against viruses and acquire insecticidal resistance
B. To strengthen the plant to grow against bacterial diseases

C. Develop the plants to grow in draught
D. Engineering plants for nutritional quality
E. Make the plants to grow in various seasons
F. Herbicide resistant plant can be made
G. Resistance against fungal pathogens
H. Engineering of plants for abiotic stress tolerance
I. Delayed ripening can be done

TYPES OF GENE TRANSFER

1. Physical gene transfer methods

a) **Electroporation:** An electric field is used to improve the permeability of the cell membrane, allowing DNA to enter. Protoplasts are typically used in plant cell electroporation, but macromolecule mobility is constrained by thick plant cell walls.

The plant material is exposed to high voltage electrical impulses while being cultured in a buffer solution containing the target or desired foreign DNA. The protoplast membrane develops tiny, transient holes as a result of the electric current, which allow DNA to flow through.

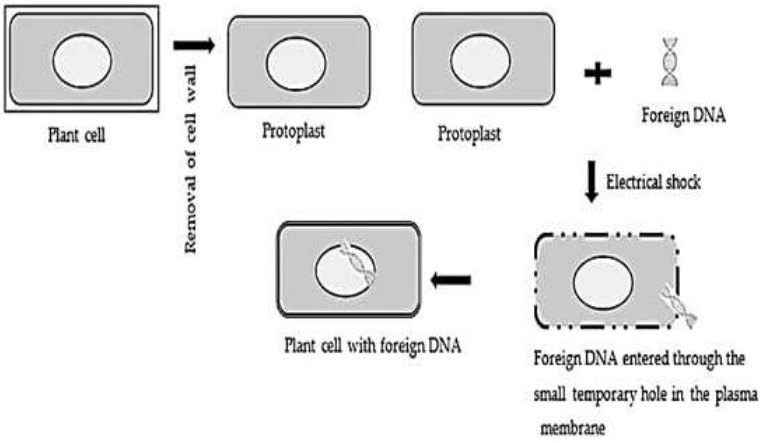

Figure 2. Electroporation.

The foreign DNA enters the cell and merges with the host genome, causing a genetic modification. The protoplasts are subsequently grown to become whole plants. This technique can be used to agricultural species when protoplast regeneration is possible.

b) **Microinjection**: A direct physical technique called microinjection involves mechanically inserting the desired DNA into a target cell. The targeted cell could be found in embryos, callus, meristems, protoplast etc. Microinjection is used to transfer cellular organelles and manipulate chromosomes. Using capillary glass micropipettes (0.5–10.0 pm tip), the DNA solution is injected straight into the cell with the help of micromanipulators of a microinjection assembly. Protoplasts are more suitable for microinjection than cells due to their lack of cell walls. Protoplasts are typically trapped in agarose, on polylysine-coated glass slides, or by being held under suction with a micropipette. After microinjection is finished, the altered cell is grown and expanded to create a transgenic plant. For example, this method has been used to create transgenic *Brassica napus* and tobacco.

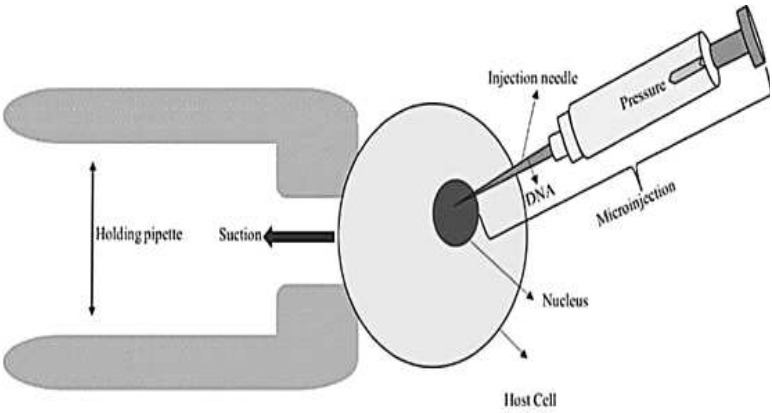

Figure 3. Microinjection Method.

c) **Gene Gun/ Biolistic transfer method**: A technique for introducing foreign DNA into plant cells is particle bombardment. The best method for transferring genes and producing transgenic plants is using gene gun. The fact that this technique can be applied to the effective transfer of DNA in both microbes and mammalian cells makes it adaptable. Sanford (1988) coined the term "biolistics" for the microprojectile bombardment approach. Biolistics is a blend of biological and ballistic concepts. Foreign DNA coated with tiny 0.2–0.7 ìm gold (or tungsten) particles is used in the transformation process to enter the target plant cells. After being put into a particle gun, the coated particles are rapidly accelerated by-

- By using pressurized helium gas
- By electro static energy released by a droplet of water exposed to a high voltage

The target could be plant cell suspensions, callus cultures, or tissues. The projectiles penetrate the membranes and cell walls of plants. The surface of the micro projectiles releases transgenes as they enter the cells for subsequent integration into the chromosomal DNA of the plant.

Utilizing plants for bombardment: Plant tissue comes in two varieties that are frequently utilized for particle bombardment:

1. Primary explants that are capable of being bombarded and then made to become embryo genic and regenerate.
2. Proliferating embryonic tissues that can be exposed to high concentrations in cultures before being permitted to grow and regenerate.

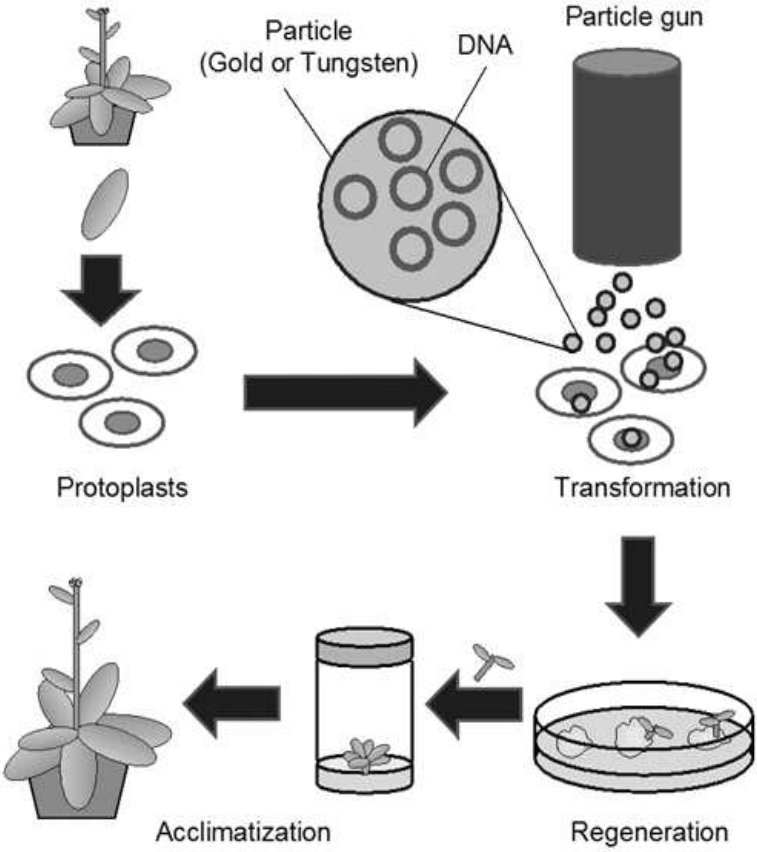

Figure 4. Gene Gun Method.

During the integrative phase, additional transgene integration can occur at or near the starting location. In the end, particle bombardment is usually linked to a high copy number at a single location. Plant regeneration may benefit from this kind of single locus.

FACTORS AFFECTING BOMBARDMENT

- **Nature of micro particles:** Inert metals such as tungsten, gold and platinum are used as micro particles to carry DNA. These particles with relatively higher

mass will have a better chance to move fast when bombarded and penetrate the tissues.

- **Nature of tissues/cells:** The target cells that are capable of undergoing division are suitable for transformation.
- **Amount of DNA:** The transformation may be low when too little DNA is used. On the other hand, too much DNA may result is high copy number and rearrangement of transgenes. Therefore, the quantity of DNA used should be balanced.
- **Environmental parameters:** Many environmental variables are known to influence particle bombardment. These factors (temperature, humidity, photoperiod etc.) influence the physiology of the plant material, and consequently the gene transfer. It is also observed that some explants, after bombardment may require special regimes of light, humidity, temperature etc.

2. Chemical gene transfer methods

a) **Polyethylene glycol (PEG)-mediated:** Polyethylene glycol (PEG)-mediated transmission in the presence of divalent cations (Ca2+), polyethylene glycol (PEG) destabilizes the plasma membrane of protoplasts, rendering it ineffective permeable to naked DNA. DNA enters the nucleus of protoplasts and integrates with their genome. The process comprises isolating and suspending protoplasts, adding plasmid DNA, and slowly adding 40% PEG-4000 (w/v) in mannitol and calcium nitrate solution. When this mixture is incubated, protoplasts undergo transformation.

Advantages of PEG-mediated Transformation

i) This technology successfully transforms many protoplasts at once.
ii) It can be used to a variety of plant species.

Limitations of PEG-mediated transformation

i) The DNA is susceptible for degradation and

rearrangement.

ii) Random integration of foreign DNA into genome may result in undesirable traits.

iii) Regeneration of plants from transformed protoplasts is a difficult task.

b) Diethyl amino ethyl (DEAE) dextran-mediated: The desirable DNA can be complexed with a high molecular weight polymer diethyl amino ethyl (DEAE) dextran and transferred. The efficiency increased to 80% when DMSO shock is given. The major limitation of this approach is that it does not yield stable trans-formants.

c) Calcium phosphate precipitation: The DNA is allowed to mix with calcium chloride solution and isotonic phosphate buffer to form DNA-calcium phosphate precipitate. When the actively dividing cells in culture are exposed to this precipitate for several hours, the cells get transformed. The success of this method is dependent on the high concentration of DNA and the protection of the complex precipitate. Addition of dimethyl sulfoxide (DMSO) increases the efficiency of transformation.

3. Agrobacterium mediated gene transfer:

The Agrobacterium system was the first effective plant transformation system, leading to a breakthrough in plant genetic engineering in 1983. The Agrobacterium is naturally occurring gram negative soil bacterium with two common species *A Tumifacience* and arhizogenes there are known as natural gene engineers for their ability to transform plants. Tumifacience produces crown galls, while rhizogenes causes hairy root infections. These bacteria have large plasmids known as root-inducing (Ri plasmid) and tumor-inducing (Ti plasmid), respectively. *A. tumefaciens'* inherent, special aptitude is largely responsible for the development of plant transformation techniques.

The Ti plasmid's transformation targets two key segments: T DNA and viral area. The T DNA portion of the Ti plasmid is transported to plant cells and integrated into the nuclear genome of cells. T DNA transfer is facilitated by virs genes, which are located in another region of the Ti plasmid. Modified Ti plasmids are created by removing unwanted Ti genes and replacing them with a foreign gene (e.g., antibiotic resistance) and a closely connected selectable marker gene. Genes inserted into plasmid cysts' T DNA region are transmitted to the plant genome. T DNA is often incorporated in low copy numbers per cell. *A. tumifacience* can only transfer genes to injured plant parts and has a limited host range. It can infect approximately 60% of gymnosperms and angiosperms. Agrobacterium-mediated transformation is preferred for dicotyledonous plant species with well-established regeneration systems. However, Agrobacterium-mediated gene transfer is not effective for monocotyledons.

In general, most of the Agrobacterium-mediated plant transformations have the following basic protocol:

a. Development of Agrobacterium carrying the co-integrate or binary vector with the desired gene
b. Identification of a suitable explant e.g. cells, protoplasts, tissues, calluses, organs
c. Co-culture of explants with Agrobacterium
d. Killing of Agrobacterium with a suitable antibiotic without harming the plant tissue
e. Selection of transformed plant cells
f. Regeneration of whole plants

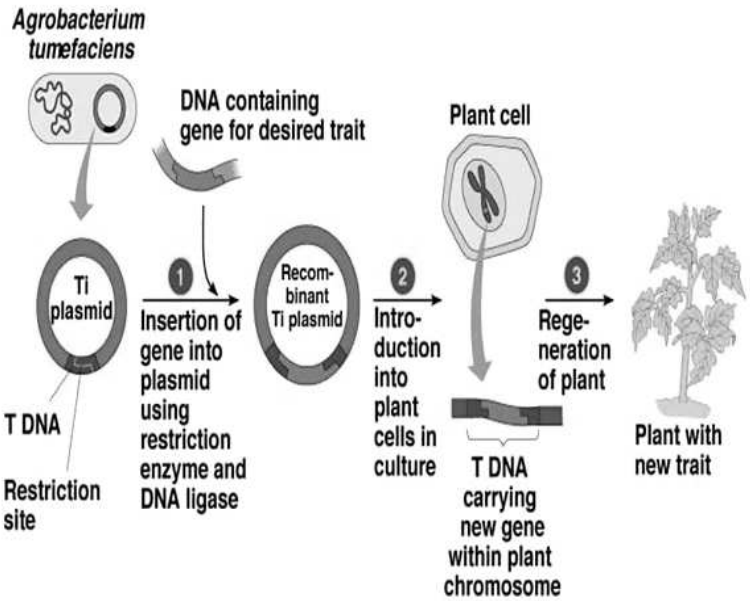

Figure 5. Agrobacterium mediated gene transfer.

ADVANTAGES

- It is a natural form of gene transfer.
- Agrobacterium can infect plant cells and tissues.
- Agrobacterium can transmit large DNA fragments efficiently
- T DNA integration is a relatively accurate process
- Gene transfer stability is excellent
- Transformed plants can be regenerated effectively.

LIMITATIONS

- Host specificity: There is a limitation of host plants for Agrobacterium, since many crop plants (monocotyledons e.g. cereals) are not infected by it.
- Inability to transfer multiple genes: The cells that regenerate more efficiently are often difficult to transform, e.g. embryonic cells lie in deep layers which

are not easy targets for Agrobacterium.
- Soma clonal variation
- Slow regeneration

REFERENCES

- *Rahangdale, S., Singh, Y., Katkani, D., & Surjaye, N. (2020). Gene Transfer Methods in Plants. 10.22271/int.book.11.*
- *Bolukbasi, E. & Aras, S. (2016). Gene transfer methods and applications in plants.*
- *Potrykus, I. (2007). Gene Transfer Methods for Plants and Cell Cultures. 198-212. 10.1002/9780470514009.ch14.*
- *Tiwari, M., Mishra, A., & Chakrabarty, D. (2022). Agrobacterium-mediated gene transfer: recent advancements and layered immunity in plants.* Planta, *256. 10.1007/s00425-022-03951-x.*
- *Kumar, K., Das, A., Sharma, S., Tribhuvan, K., Janghel, D., & Srivastava, H. (2019). Agrobacterium Mediated Gene Transfer in Plants: An Extension and Modification of Natural Process.*
- *Alsaggar, M. & Liu, D. (2015). Physical Methods for Gene Transfer.* Advances in genetics, *89. 1-24. 10.1016/bs.adgen.2014.10.001.*

Chapter-9

Implementation of Transgenic Plants and PCR Technology for crop Improvement.

Taznim Hossain[1], Sasmita Das[1], Gyanarasmi Swain[2] and Lakshmi Singh[1*]

[1]Department of Botany, College of Basic Science and Humanities, Odisha University of Agriculture and Technology, Bhubaneswar-751003, Odisha, India.
[2]Department of Botany, Regional Institute of Education, Bhubaneswar, Odisha, India.
lakshmisingh@ouat.ac.in

ABSTRACT

Unlike conventional plant breeding methods, transgenic crops are developed through genetic engineering and have proven to meet the food security needs of the growing global population. To date, many transgenic plants have been commercialized, with many more undergoing research trials to address their biosafety concerns. Genetically modified plants also offer enhanced nutritional quality in food alongside their high resistance traits. The development of PCR has revolutionized the field of biological research. Over time, PCR technologies have advanced significantly. The PCR technique is now applied in almost every scientific area and is extremely beneficial. Some of its applications include the production of innovative biomedicines, early detection of genetic disorders, clinical treatments and also in screening transgenic plants.

KEYWORDS: Transgenic plants, crop improvement, genetically modified plants, PCR.

INTRODUCTION

Transgenic plants or genetically engineered plants are those plants in which their gene(s) have been modified by introducing foreign genes (transgenes) from another plant, which is done based on our desired traits. This artificial insertion of a foreign gene is done by various biotechnological methods. These transgenic plants carry the desired genes and show economic and commercial importance, such as enhanced quality, greater yield, resistance against biotic and abiotic stress, in vaccine production, etc. In 1982, the 1st commercial transgenic plant i.e., a virus-resistant tobacco plant, was reported. Some of the examples of transgenic plants are golden rice, roundup ready soybean, etc. In recent years, various pharmaceutical products have been produced with the help of transgenic plants, which is done by using transgenic plants as bioreactors. Transgenic seeds or fruits are now easily transported and stored without being degraded. Such seeds are stored for further hybridization in crop breeding programs based on the need of the products to be produced. Several immunotherapeutic drugs are being produced using these bioreactors or biofactories. Biodegradable plastics are also being prepared using PHB (Polyhydroxybutyrate), a compound derived from transgenic plants. Various antibodies, edible vaccines (1990) started to appear commercially, which provided immunization. IgG, IgA, and serum albumin genes were successfully incorporated into plants. Polymerase chain reaction (PCR) plays a critical role in the analysis of transgenic genes present in transgenic plants. It is an *in-vitro* molecular technique used to amplify the no. of a specific DNA region, including the transgenes, and confirms the presence of transgenes in a short period, and helps in the understanding of transgene function and potential silencing. Identification and detection of the transgenes in transgenic plants have been reported globally. The PCR technique uses an internal standard i.e., endogenous gene which acts as a

binding site for one primer and the other primer is designed to bind to the specific sequence of the target DNA sequence of the transgenic plant, thus differentiating the transgenic plants from the non-transgenic ones.

1. IMPORTANCE OF TRANSGENIC IN CROP IMPROVEMENT

Transgenics is a technology that allows the introduction of specific genes from foreign organisms to manipulate traits like quality, yield, and resistance (drought, salinity, and pest infections) shown in **Figure 1.** The importance of transgenic in response to resistance to pests and harmful diseases to improve food production are in high demand and work towards sustainability. Here are some important implications of transgenics.

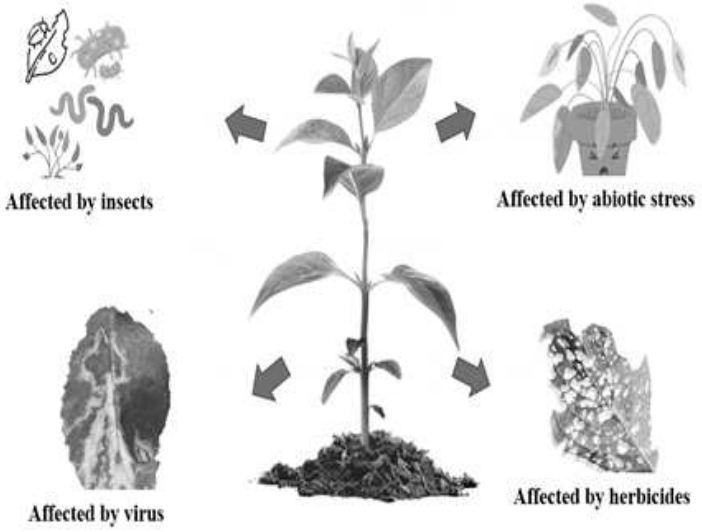

Figure 1. shows the plants affected by different kinds of both biotic and abiotic stresses.

1.1 INSECT-RESISTANT TRANSGENIC PLANTS

Bacillus thuringiensis contains the *Bt/cry* gene, which

encodes beta and delta endotoxins, which are proteinaceous toxins that destroy the gut epithelium of harmful insects. There are mainly four groups of *cry* genes based on their toxin-producing activities- *cry* I, *cry* II, *cry* III, and *cry* IV. The *Bt* genes are isolated and introduced into the Ti-plasmid of *A. tumefaciens* and are finally introduced into the plant cells. *Bt* cotton was commercialised in India on March 2003. Some other examples include *Bt* corn, *Bt* maize, etc.

1.2 VIRUS-RESISTANT TRANSGENIC PLANTS

Plant viruses cause harm to a large no. of plants resulting in the decrease of crop yield. Such plants are produced by introducing CP (coat protein) gene of TMV into the desired plant. Some of the examples include tomato (using CP gene of TMV), papaya (using CP gene of PRSV), etc.

1.3 HERBICIDE-RESISTANT TRANSGENIC PLANTS

These plants use different mechanisms for resistance against glyphosate, sulphoylurea and imidazolinone herbicides. For resistance against glyphosate, RPSPS genes were introduced in plants, and similarly, a mutant *als* gene was used to provide resistance against the other two herbicides mentioned above.

1.4 NUTRITIONAL CONTENT IN RESPONSE TO TRANSGENICS

Vitamin A is a very important fat-soluble vitamin needed by our body. Its deficiency causes night blindness and skin disorders. This deficiency is mainly found in children. Rice, being the staple food in many countries, has been found to contain low vitamin A levels. Thus, golden rice was engineered, which contained high levels of beta-carotene, a precursor for Vit-A for overcome the problems regarding Vitamin A deficiency.

2. HOW ARE TRANSGENIC PLANTS PRODUCED?

1) First, we need a piece of DNA that encodes our desired trait, including a promoter which helps in the gene expression. This all happens in a plasmid vector that replicates in a bacterial cell to make large no. of DNA copies.

2) Now, we need a way to select the cells containing our DNA containing the plasmids. This is achieved by adding a selectable marker, most commonly an antibiotic resistance gene.

3) Then we need to incorporate the target DNA inside the plant, which is done by:

a) **BRUTE FORCE METHOD: A GENE GUN**-Here, a gene gun fires the DNA coated gold particles (including promoter, sequence for the desired trait and a selection marker) into the plant cells.

b) **AGROBACTERIUM MEDIATED GENE TRANSFER**- *Agrobacterium tumefaciens* is a gram-negative soil bacterium. It causes crown gall disease by infecting the wound sites of plant, caused as a result of gene transfer. It has Ti-plasmid which consists of T-DNA (containing the genes to be transferred) and vir genes (help in gene regulation and T-DNA processing) into the plant naturally. Thus T-DNA expresses itself in the transgenic plants.

4) At last, the transformed plant that has the integrated DNA is grown under sterile conditions using tissue culture method.

3. PCR AND ITS APPLICATIONS IN TRANSGENIC CROPS

As the global population rises, the demand for the production of transgenic plants is also increasing rapidly. With the rate of new transgenic plants being introduced on the rise, there is a need for a molecular tool to help us distinguish between transgenic plants containing exogenous or foreign genes and non-transgenic plants. Several modern tools have been introduced to detect these foreign genes, but most of them are time-consuming and expensive, making them less convenient for our purpose. Polymerase Chain Reaction (PCR) is one of the widely used analytical tools for detecting foreign genes. PCR, being a fast, simple, highly specific, sensitive, reliable, and inexpensive method, has been found suitable to meet our needs. Detecting transgenes becomes crucial for screening a large number of transgenic plants due to the interference of the endogenous genes of plants with transgenes.

3.1 PCR TECHNIQUES

PCR techniques have been used in transgene detections and analysis of transgenic plants like rice, chickpea, soy, maize, etc. These techniques are helpful in amplifying and detecting specific sequences such as regulatory gene or structural genes in the transgene. PCR can also be combined with other techniques like ELISA, southern blotting, northern blotting, etc. for enhanced screening of transgenes.

1)PRIMER DESIGN- Binds with the specific sequences of the transgene.	**2)AMPLIFICATION**- Target DNA sequence is amplified using primers to detect transgene.	**3) QUALITATIVE PCR**- Confirms whether transgenes are present or not.	**4) qPCR**- Quantifies the copy no. and expression level in transgenic plants.

3.2 APPLICATIONS OF PCR IN TRANSGENIC CROP

There are two main ways to detect the presence of transgenic

genes in plants. They are as follows:

3.2.1 QUANTITATIVE PCR (qPCR)

This method is used to determine the measure of the transgene's copy number precisely along with its level of expression that have been introduced into the genome of plant. Here, the target sequence of transgene is amplified and compared with endogenous reference gene. Then a standard curve is produced to quantify copy no. of transgenes. Expression levels are quantified using mRNA levels. It is a more efficient substitute to southern blotting for quantifying transgenes to screen large no. of plants. It also tells us about the effects of transgenes regarding the stability, morphological traits, etc. in the plant genome. RT-PCR along with qPCR is also used for this method where cDNAs are produced using mRNA which are amplified and then quantified **(Figure 2)**.

3.2.2 QUALITATIVE PCR

This method is divided into two ways: screening and event-specific PCR method. In screening, sequences that are common in transgenic plants are monitored, such as the CaMV 35S promoter of CMV (cauliflower mosaic virus). In the event-specific method, transgenic events are detected to differentiate between the transgene and the plant's genome. Here, designed primers bind to the specific DNA sequence of the plant sample and amplify it. The PCR will produce a detectable product, indicating the presence of the sequence in the sample and vice versa. Using the designed primer that binds to a specific sequence of the transgene helps in differentiating the transgene from the plant genome **(Figure 2)**.

Isolated mRNA

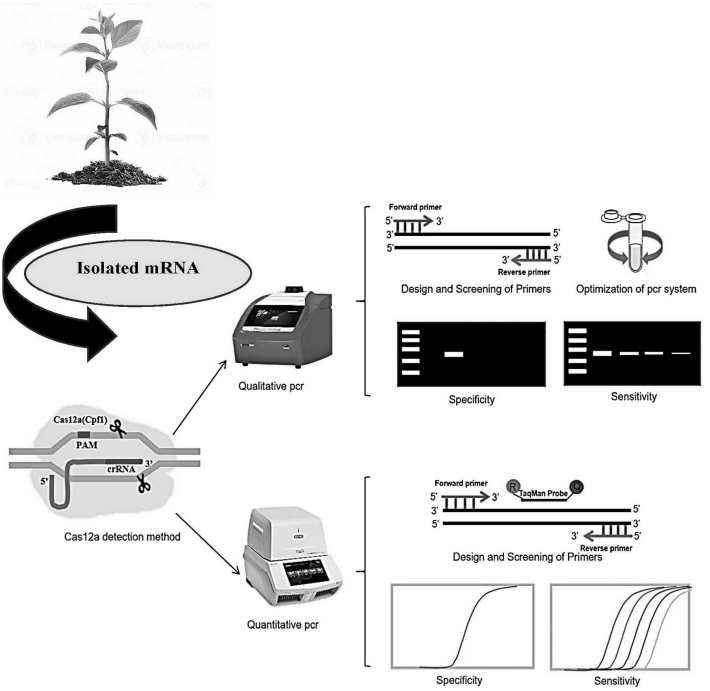

Figure 2. Shows the qualitative and quantitative PCR for transgenic crop improvement

REFERENCES

- *Rani, S. J., & Usha, R. (2013). Transgenic plants: Types, benefits, public concerns and future.* Journal of Pharmacy Research, 6*(8), 879-883.*
- *Marone, D., Mastrangelo, A. M., & Borrelli, G. M. (2023). From transgenesis to genome editing in crop improvement: applications, marketing, and legal issues.* International Journal of Molecular Sciences, 24*(8), 7122.*
- *Khehra, N., Padda, I., & Swift, C. (2023). Polymerase chain reaction (PCR).* StatPearls.
- *Singh, J., Birbian, N., Sinha, S., & Goswami, A. (2014). A criti-*

cal review on PCR, its types and applications. Int. J. Adv. Res. Biol. Sci, 1*(7), 65-80.*

- *Lipp, M., Shillito, R., Giroux, R., Spiegelhalter, F., Charlton, S., Pinero, D., & Song, P. (2005). Polymerase chain reaction technology as analytical tool in agricultural biotechnology.* Journal of AOAC International, 88*(1), 136-155.*
- *ªule, A. R. I., & GÖZÜKIRMIZI, F. G. N. Determination of Transgenic Plants Using Polymerase Chain Reaction.* technology, *2, 331.*
- *Lee, D. K., Seok, S. J., Jang, I. C., Nahm, B. H., & Kim, J. K. (1998). Triprimer-PCR method: rapid and reliable detection of transgenes in transgenic rice plants.* Molecules and cells, 8*(1), 101-106.*
- *Firke, P.K., & Randhawa, G.J. (2005). Polymerase Chain Reaction for Evaluation! Detection of Transgenic Planting Material.* Indian Journal of Plant Genetic Resources, 18*(01), 143-143.*
- *De Saeger, J., Park, J., Thoris, K., De Bruyn, C., Chung, H. S., Inzé, D., & Depuydt, S. (2022). IMPLANT: a new technique for transgene copy number estimation in plants using a single end-point PCR reaction.* Plant Methods, 18*(1), 132.*
- *Arý, S., Bayroviç, K., Gürel, F., & Gözükýrmýzý, N. (1995). Determination of transgenic plants using polymerase chain reaction.*
- *Horsch, R.B., Rogers, S.G., & Fraley, R.T. (1985). Transgenic plants. In* Cold Spring Harbor symposia on quantitative biology *(Vol. 50, pp. 433-437). Cold Spring Harbor Laboratory Press.*
- *Larrick, J.W., Yu, L., Chen, J., Jaiswal, S., & Wycoff, K. (1998). Production of antibodies in transgenic plants.* Research in immunology, 149*(6), 603-608.*

Chapter-10

Role of DNA Markers (RFLP, RAPD, SSR) in Marker-Assisted Selection for Crop Improvement and Biotechnology Regulation

Radharani Singh[1], Sudip Das[2], Lelina Dash[3] and Dr. Subrat Kumar Kar[4]*

[1]Department of Biotechnology, Fakir Mohan University, Balasore, Odisha, India.

[2]Department of Plant Physiology and Biochemistry, Bihar Agricultural University, Sabour, Bhagalpur, India.

[3]Department of Biotechnology, Maharaja Sriram Chandra Bhanja Deo University, Baripada, Odisha, India.

[4]Department of Biotechnology, MITS School of Biotechnology, Bhubaneswar, Odisha, India, 751024.*

subratkar14@gmail.com

ABSTRACT

DNA markers have transformed the landscape of plant breeding and biotechnology by enabling more accurate and efficient identification of genetic traits associated with agronomic importance. Marker-Assisted Selection (MAS), which leverages these markers, facilitates early selection of desirable genotypes, reducing breeding cycles and improving the precision of crop improvement programs. Among the diverse types of DNA markers, Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), and Simple Sequence Repeats (SSR) have been pivotal in the genetic analysis of crops due to their reliability, informativeness, and applicability across a wide range of species. This chapter explores the principles, advantages, and limitations of RFLP, RAPD, and SSR markers and highlights their specific

contributions to MAS, such as in the identification of disease resistance genes, mapping of quantitative trait loci (QTLs), and evaluation of genetic diversity. Moreover, the role of these markers in the regulatory frameworks governing genetically modified organisms (GMOs) and plant variety protection is discussed, emphasizing their importance in traceability, biosafety compliance, and intellectual property rights. With continued advancements in molecular technologies, these markers remain essential tools, especially in regions where high-throughput sequencing technologies are not yet widely accessible.

KEYWORDS: DNA markers, Marker-Assisted Selection (MAS), Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Simple Sequence Repeats (SSR), crop improvement, genetic mapping, QTL analysis, Plant biotechnology, GMO detection, Molecular markers.

INTRODUCTION

Plant breeding has evolved from traditional phenotypic selection to incorporate molecular tools, enhancing the precision and efficiency of developing improved crop varieties. Marker-Assisted Selection (MAS) is a pivotal molecular breeding technique that utilizes DNA markers linked to specific traits, facilitating the selection of desirable genotypes without the need for phenotypic expression. This chapter delves into the principles, methodologies, applications, and challenges of MAS, providing a comprehensive overview for researchers and practitioners in the field. The advent of molecular biology has introduced DNA markers as powerful tools in plant breeding. These markers enable the identification of genetic variations associated with specific traits, allowing breeders to select for these traits without relying solely on phenotypic expression.

RFLP, RAPD, and SSR markers have been pivotal in this transformation, each offering unique advantages and limitations.

Modern agriculture faces numerous challenges, including the need for higher crop productivity, resistance to biotic and abiotic stresses, and sustainable agricultural practices. Traditional plant breeding, though effective, is time-consuming and less precise. Marker-Assisted Selection (MAS), an approach rooted in molecular genetics, enhances the efficiency and accuracy of crop improvement programs. DNA markers are central to MAS, enabling indirect selection of desired traits based on genotype rather than phenotype.

Among the many molecular markers developed, Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), and Simple Sequence Repeats (SSR) have played foundational roles in plant genetics and breeding. This chapter explores the characteristics, applications, and significance of these markers in crop improvement and biotechnology regulation.

The integration of molecular biology into plant breeding has significantly transformed traditional methodologies, enabling more precise and efficient crop improvement strategies. Central to this transformation is the development and application of DNA markers (specific sequences in the genome that serve as indicators of genetic variation associated with particular traits). These markers facilitate the identification and selection of desirable traits at the genotypic level, independent of phenotypic expression, which can be influenced by environmental factors and developmental stages. This approach, known as Marker-Assisted Selection (MAS), has become a cornerstone in modern plant breeding, offering a means to accelerate the development of crop varieties with enhanced traits such as disease resistance, abiotic stress tolerance, and improved nutritional quality.

THE EVOLUTION OF PLANT BREEDING THROUGH MOLECULAR TOOLS

Traditional Breeding Challenges and the Need for MAS

Traditional plant breeding, while foundational, often faces limitations in precision and efficiency, particularly when dealing with complex traits controlled by multiple genes. These traits, such as drought tolerance or disease resistance, are typically expressed late in the plant's life cycle, making phenotypic selection challenging and time-consuming. Additionally, traits with low heritability can be difficult to improve through conventional methods. MAS addresses these challenges by allowing breeders to select for traits based on genetic information, thereby enhancing the accuracy and speed of breeding programs.

Marker-Assisted Selection (MAS): A Paradigm Shift in Breeding

Traditional plant breeding methods, while effective, are often time-consuming and less precise, particularly when dealing with complex traits controlled by multiple genes. MAS addresses these challenges by utilizing molecular markers to select for desirable traits based on genotype, thus bypassing the limitations of phenotypic selection. This approach has proven advantageous in various aspects:

- **Early Detection of Traits:** MAS allows for the identification of traits expressed late in the plant's life cycle, such as disease resistance or fruit quality, at early developmental stages like seedling or nursery phases.
- **Identification of Recessive Alleles:** MAS can detect recessive alleles even in heterozygous conditions, expediting the selection process for traits that are otherwise challenging to identify phenotypically.
- **Gene Pyramiding:** The technique facilitates the accumulation of multiple genes for resistance to specific

pathogens or pests within the same cultivar, enhancing durability and effectiveness.

- **Screening Low Heritability Traits:** MAS is particularly effective for traits with low heritability, such as resistance to abiotic stresses like drought or salinity, which are difficult to evaluate through traditional phenotypic methods.

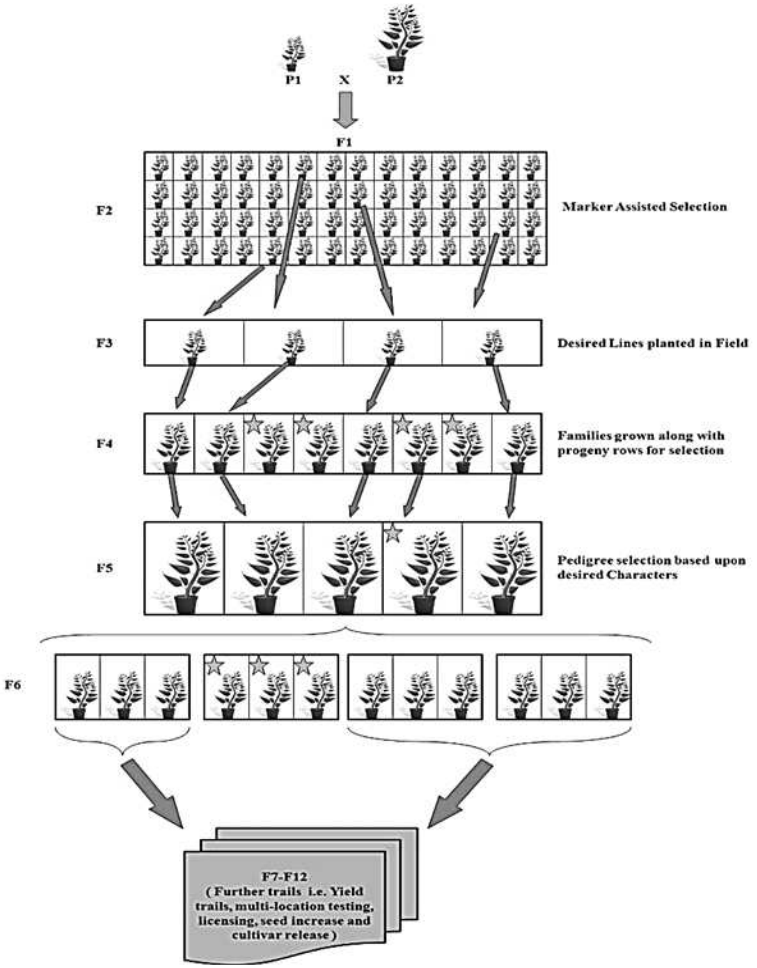

Figure 1. Marker-Assisted Selection (MAS).

Principles of Marker-Assisted Selection

MAS operates on the concept of genetic linkage, where molecular markers are associated with genes or Quantitative Trait Loci (QTLs) that control specific traits. The primary steps involved in MAS include:

- **Identification of Molecular Markers:** Utilizing markers such as Simple Sequence Repeats (SSRs), Single Nucleotide Polymorphisms (SNPs), and Random Amplified Polymorphic DNA (RAPD) to identify genetic variations associated with traits of interest.
- **Marker-Trait Association:** Establishing a correlation between the identified markers and the phenotypic traits through genetic mapping and QTL analysis. Selection of Desirable Genotypes: Employing the identified markers in breeding populations to select individuals carrying favourable alleles, thereby accelerating the breeding process.

DNA Markers in Marker-Assisted Selection

The application of DNA markers has revolutionized plant breeding by facilitating marker-assisted selection (MAS), enabling breeders to identify and select desirable traits at the molecular level rather than relying solely on phenotype. Among the various molecular markers developed, Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), and Simple Sequence Repeats (SSR) have played pivotal roles in crop genetics and breeding programs.

A. Restriction Fragment Length Polymorphism (RFLP)

RFLP markers detect variations in DNA fragment lengths resulting from polymorphisms in restriction enzyme recognition sites. This technique involves digesting DNA with specific enzymes, separating the fragments via gel electrophoresis, and detecting the variations through hybridization with labeled

probes. RFLPs are co-dominant markers, providing high-resolution mapping of genes associated with traits such as disease resistance and yield components. However, the method is labor-intensive and requires high-quality DNA samples. Provides high reproducibility and is co-dominant, allowing for the detection of both alleles in a heterozygote. However, it requires a large amount of high-quality DNA and is labor-intensive, making it less suitable for large-scale applications. RFLP was one of the earliest DNA marker technologies employed in genetic analysis and plant breeding. It is based on the variation in DNA fragment lengths produced by the digestion of genomic DNA with specific restriction enzymes, followed by hybridization with labeled probes that target specific sequences (Botstein et al., 1980). RFLPs are co-dominant markers, which means they can distinguish between homozygous and heterozygous genotypes, providing precise genetic information. This marker system was instrumental in constructing the first genetic maps of several plant species, including maize and rice (Helentjaris et al., 1986; McCouch et al., 1988).

However, RFLP has significant limitations, including labor-intensive protocols, the requirement for large amounts of high-quality DNA, and reliance on radioactive labeling techniques, which have led to a decline in its routine use (Tanksley et al., 1989).

B. Random Amplified Polymorphic DNA (RAPD)

RAPD markers utilize short, random primers to amplify DNA segments via PCR, generating polymorphic patterns. This technique does not require prior sequence information, making it applicable to a wide range of species. RAPDs are dominant markers and are particularly useful for assessing genetic diversity, identifying cultivars, and constructing genetic maps. Despite their advantages, RAPDs suffer from low reproducibility and are less effective in distinguishing

heterozygous individuals. A PCR-based technique that is simple and cost-effective, RAPD markers are useful for initial screenings and genetic diversity studies. Despite their advantages, RAPDs can be less reproducible and may require optimization for each new species or population. RAPD markers were introduced as a more rapid and less technically demanding alternative to RFLP. This technique uses short, arbitrary primers in polymerase chain reactions (PCR) to amplify random segments of genomic DNA, generating unique fingerprints for different genotypes. RAPD is particularly advantageous due to its simplicity, low cost, and the fact that it does not require prior knowledge of the genome. Despite these advantages, RAPDs are dominant markers, which means they cannot distinguish between homozygous dominant and heterozygous genotypes. Moreover, they suffer from reproducibility issues across different laboratories and experimental conditions, limiting their utility in precise breeding applications.

C. Simple Sequence Repeats (SSR)

SSR markers, also known as microsatellites, are short, repetitive DNA sequences scattered throughout the genome. They are highly polymorphic and co-dominant, making them ideal for genetic diversity studies, marker-assisted backcrossing, and fine mapping of quantitative trait loci (QTLs). SSRs offer high reproducibility and are widely used in various crops, including rice, maize, and legumes. Characterized by high polymorphism, co-dominant inheritance, and reproducibility, SSR markers are widely used in various crops. They are particularly valuable for constructing genetic maps and conducting marker-trait association studies. However, the development of SSR markers can be time-consuming and may not be feasible for all species. SSR markers, also known as microsatellites, consist of short, tandemly repeated DNA sequences (e.g., (CA)n, (AT)n) that are widely dispersed

throughout the genome. These regions are highly polymorphic due to the variability in the number of repeat units, making them exceptionally useful for genetic mapping and diversity studies. SSRs are co-dominant, highly reproducible, and locus-specific, and they offer high levels of polymorphism, which significantly enhances their resolution in MAS. SSR markers have been extensively used in major crops such as wheat, rice, maize, and soybean for trait mapping, genotype identification, and marker-assisted backcrossing. Their efficiency in detecting polymorphisms and ease of automation make SSRs one of the most valuable tools in modern plant breeding.

Comparative Analysis of RFLP, RAPD, and SSR

Marker Type	Co-dominant	Reproducibility	Polymorphism Level	Application Areas
RFLP	Yes	High	Moderate	Gene mapping, QTL analysis
RAPD	No	Low	High	Genetic diversity, cultivar identification
SSR	Yes	High	Very High	Marker-assisted backcrossing, fine mapping

ADVANCEMENTS AND APPLICATIONS IN CROP IMPROVEMENT

The application of MAS has led to significant advancements in crop improvement, including the development of varieties with enhanced resistance to diseases and pests, improved nutritional content, and better adaptation to environmental stresses. The ability to perform early selection and track multiple genes simultaneously has accelerated breeding cycles and increased the precision of trait introgression. Moreover, MAS has facilitated the conservation and utilization of genetic resources, enabling the identification and incorporation of beneficial traits from wild relatives and underutilized species. This is particularly important in the context of climate change, where the need for resilient and adaptable crop varieties is

paramount.

Applications of Marker-Assisted Selection

MAS has been instrumental in various aspects of crop improvement:

- **Disease Resistance:** Facilitating the introgression of resistance genes into elite cultivars, thereby enhancing disease resistance without the need for pathogen exposure (Ribaut & Ragot, 2006).
- **Abiotic Stress Tolerance:** Identifying and selecting for genes associated with drought, salinity, and heat tolerance, crucial for adapting crops to changing climatic conditions.
- **Quality Traits:** Improving nutritional content, processing quality, and other consumer-preferred traits through targeted selection.
- **Yield Improvement:** Utilizing MAS to select for yield-related QTLs, thereby enhancing productivity in diverse environmental conditions.

Strategies in Marker-Assisted Selection

MAS can be implemented through various strategies:

- **Marker-Assisted Backcrossing (MABC):** A method to transfer specific genes or QTLs from a donor to a recurrent parent, minimizing linkage drag and accelerating the recovery of the recurrent parent genome.
- **Marker-Assisted Recurrent Selection (MARS):** Involves multiple cycles of selection and recombination to accumulate favourable alleles from different sources, enhancing the genetic base of the breeding population.
- **Gene Pyramiding:** The simultaneous incorporation of multiple genes conferring resistance to different pathogens or stresses, providing broad-spectrum and durable resistance.

APPLICATIONS IN CROP IMPROVEMENT

Disease Resistance

Markers linked to disease resistance genes enable the development of cultivars with enhanced resistance to pathogens. For instance, SSR markers have been associated with resistance to Ascochyta blight in chickpea, facilitating the breeding of resistant varieties.

Abiotic Stress Tolerance

Identifying markers linked to traits like drought tolerance allows for the development of crops that can withstand adverse environmental conditions. RFLP markers have been employed to map genes associated with drought resistance in various crops.

Yield Improvement

Marker-assisted selection aids in the introgression of yield-related traits from wild relatives into elite cultivars. SSR markers have been instrumental in identifying QTLs associated with yield components in crops like rice and maize.

ADVANTAGES OF MARKER-ASSISTED SELECTION

MAS offers several benefits over traditional breeding methods:

- **Efficiency:** Accelerates the breeding process by enabling early selection at the seedling stage.
- **Precision:** Increases the accuracy of selecting for complex traits controlled by multiple genes.
- **Cost-Effectiveness:** Reduces the need for extensive field trials and phenotypic evaluations, leading to cost savings in breeding programs.

CHALLENGES AND LIMITATIONS

Despite its advantages, MAS faces certain challenges:

- **High Initial Costs:** The establishment of molecular laboratories and procurement of genotyping equipment can be expensive.

- **Complex Traits:** Traits controlled by multiple QTLs may require advanced strategies like Genomic Selection (GS) to effectively capture genetic variation.
- **Marker Availability:** The lack of well-characterized markers for certain crops or traits can limit the application of MAS.

CONCLUSION

The integration of molecular markers such as RFLP, RAPD, and SSR has transformed traditional plant breeding into a more precise and efficient science. These markers have enabled breeders to identify and select for desirable traits at the DNA level, significantly improving the speed and accuracy of crop improvement programs. Among them, SSR markers have emerged as the most widely used in modern MAS due to their high reproducibility, polymorphism, and co-dominant inheritance. Beyond their utility in breeding, molecular markers also play a critical role in the regulatory frameworks surrounding biotechnology, aiding in the monitoring and safe deployment of genetically modified organisms. Their dual function in both crop development and biosafety underscores their broad relevance in modern agriculture.

As the global community faces mounting challenges related to food security, climate change, and sustainable agriculture, the continued advancement and integration of molecular technologies into breeding programs will be essential. Marker-Assisted Selection, supported by robust molecular tools, stands at the forefront of efforts to develop resilient, high-yielding, and environmentally sustainable crop varieties. The future of plant breeding lies in the ongoing refinement and application of these molecular tools, which not only build upon the foundations laid by earlier technologies like RFLP and RAPD but also point toward a more sustainable and secure agricultural future.

REFERENCES

- *Akagi, H., Yokozeki, Y., Inagaki, A., & Fujimura, T. (1996). Microsatellite DNA markers for rice chromosomes.* Theoretical and Applied Genetics, 93*(7), 1071–1077.*
- *Eathington, S. R., Crosbie, T. M., Edwards, M. D., Reiter, R. S., & Bull, J. K. (2007). Molecular markers in a commercial breeding program.* Crop Science, 47*(S3), S-154–S-163. https://doi.org/10.2135/cropsci2007.03.0106*
- *Gupta, P. K., Varshney, R. K., Sharma, P. C., & Ramesh, B. (1996). Molecular markers and their applications in wheat breeding.* Plant Breeding, 115*(3), 255–271.*
- *Hasan, M. M., et al. (2021). Advancements in maize genomic tools for enhanced crop breeding.* Maize Genomics and Genetics, 4*(2), 1–12. https://doi.org/10.3934/mgg.2021.2.1*
- *Hasan, N., Choudhary, S., Naaz, N., Sharma, N., & Laskar, R. A. (2021). Recent advancements in molecular marker-assisted selection and applications in plant breeding programmes.* Journal of Genetic Engineering and Biotechnology, 19*(1), 128. https://doi.org/10.1186/s43141-021-00231-1*
- *He, Z., Zhang, H., Gao, F., Hou, J., Li, H., & Bai, G. (2014). Advances in molecular marker development and applications in crop breeding.* Molecular Breeding, 34*(4), 1–15. https://doi.org/10.1007/s11032-014-0041-1*
- *Helentjaris, T., Weber, D., & Wright, S. (1986). Use of RFLP markers for analyzing genetic linkage in maize.* Theoretical and Applied Genetics, 72*(6), 761–769.*
- *Jones, C. J., Edwards, K. J., Castaglione, S., Winfield, M. O., Sala, F., van de Wiel, C., Bredemeijer, G., Vosman, B., Matthes, M., Daly, A., Brettschneider, R., Bettini, P., Buiatti, M., Maestri, E., Malcevschi, A., Marmiroli, N., Aert, R., Volckaert, G., Rueda, J., ... Karp, A. (1997). Reproducibility testing of RAPD, AFLP, and SSR markers in plants by a network of European laboratories.* Molecular Breeding, 3, *381–390.*

- *Kumar, S. (2011). Towards marker-assisted selection in pulses: A review.* Plant Breeding, 130*(3), 297–307. https://doi.org/10.1111/j.1439-0523.2011.01851.x*
- *Miklas, P. N., Kelly, J. D., Beebe, S. E., & Blair, M. W. (2006). Marker-assisted selection for disease resistance in common bean.* Euphytica, 147*(1), 35–47. https://doi.org/10.1007/s10681-006-4600-3*
- *Morgante, M., & Olivieri, A. M. (1993). PCR-amplified microsatellites as markers in plant genetics.* The Plant Journal, 3*(1), 175–182.*
- *Ribaut, J.-M., & Hoisington, D. A. (1998). Marker-assisted selection: New tools and strategies.* Trends in Plant Science, 3*(6), 236–239. https://doi.org/10.1016/S1360-1385(98)01223-1*
- *Ribaut, J.-M., & Ragot, M. (2006). Marker-assisted selection: New tools and strategies.* Trends in Plant Science, 11*(6), 213–220. https://doi.org/10.1016/j.tplants.2006.04.003*
- *Tanksley, S. D., Ganal, M. W., & Martin, G. B. (1989). RFLP mapping in plant breeding: New tools for an old science.* Bio/Technology, 7*, 257–264.*
- *Taran, B., Michaels, T. E., & Pauls, K. P. (2001). Genetic mapping of traits in common bean.* Plant Breeding, 120*(4), 346–352.*
- *Tautz, D. (1989). Hypervariability of simple sequences as a general source for polymorphic DNA markers.* Nucleic Acids Research, 17*(16), 6463–6471.*
- *Torres, A. M., Weigand, F., Palomino, C., Moreno, M. T., Cubero, J. I., & Gil, J. (2010). Marker-assisted selection: A strategy for the improvement of drought tolerance in crops.* Plant Breeding, 129*(3), 235–242. https://doi.org/10.1111/j.1439-0523.2009.01704.x*
- *Williams, J. G. K., Kubelik, A. R., Livak, K. J., Rafalski, J. A., & Tingey, S. V. (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers.* Nucleic Acids Research, 18*(22), 6531–6535.*