



OPTIMIZED PROTOCOLS FOR CALLUS CULTURE, IN VITRO PROPAGATION, AND MOLECULAR PROFILING OF PLANTS

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ABSTRACT

Plant tissue culture technologies have revolutionized modern plant biotechnology by enabling rapid clonal multiplication, conservation of elite germplasm, and production of disease-free planting material. Callus culture, in vitro propagation, and molecular profiling together provide an integrated platform for plant improvement and genetic stability assessment. Optimized protocols involving precise regulation of plant growth regulators, explant selection, and culture conditions significantly enhance regeneration efficiency and reduce somaclonal variation. Molecular profiling tools such as RAPD, ISSR, SSR, and sequencing-based approaches ensure genetic fidelity and reveal true-to-type regenerated plants. This review-based research paper highlights optimized methodologies for callus induction, plant regeneration, and molecular characterization to improve reproducibility, scalability, and genetic stability in plant tissue culture systems.

Keywords: - Callus culture, micropropagation, somatic embryogenesis, plant growth regulators, molecular markers, genetic fidelity

i. INTRODUCTION

Plant biotechnology has emerged as one of the most transformative branches of modern biological sciences, offering innovative solutions to global challenges in agriculture, horticulture, forestry, and environmental conservation. Among its various techniques, plant tissue culture has gained remarkable importance due to its ability to regenerate whole plants from small tissue segments under controlled laboratory conditions. This phenomenon is based on the principle of cellular totipotency, which states that every living plant cell contains the genetic potential to develop into a complete organism when provided with appropriate nutritional and hormonal conditions. This unique property forms the foundation for advanced techniques such as callus culture, *in vitro* propagation, and molecular profiling of regenerated plants.

Callus culture represents one of the earliest and most critical stages in plant tissue culture systems. A callus is an unorganized, dedifferentiated mass of plant cells that develops when an explant is cultured on a nutrient medium supplemented with specific plant growth regulators. The induction of callus is a complex physiological process influenced by several factors, including the genotype of the plant, the type of explant used, the composition of the culture medium, and environmental conditions such as temperature, light, and humidity. In most cases, auxins such as 2,4-dichlorophenoxyacetic acid (2,4-D) play a central role in initiating callus formation, while cytokinins are often involved in promoting cell division and differentiation. The balance between these two classes of hormones determines the nature of the callus and its subsequent developmental potential.

In recent years, optimization of callus induction protocols has become a major focus of research in plant biotechnology. Researchers aim to develop highly reproducible methods that maximize callus yield while maintaining genetic stability. This is particularly important because prolonged callus culture can sometimes lead to somaclonal variation, which refers to genetic or epigenetic changes occurring in cultured cells. Although such variations can be useful for plant breeding and genetic improvement, they are often undesirable in micropropagation systems where true-to-type plants are required. Therefore, optimizing culture conditions and minimizing variation is a key objective in modern tissue culture research.

Following callus formation, *in vitro* propagation or micropropagation is widely used for the rapid multiplication of plant material. This technique involves the aseptic culture of plant tissues or organs on nutrient media under controlled environmental conditions. Micropropagation is generally divided into four distinct stages: initiation, multiplication, rooting, and acclimatization. During the initiation stage, a sterile explant is introduced into the culture medium. The multiplication stage involves the rapid proliferation of shoots, often enhanced by cytokinins such as benzylaminopurine (BAP). In the rooting stage, auxins such as indole-3-butyric acid (IBA) are used to stimulate root development. Finally, acclimatization ensures the successful transfer of plantlets from *in vitro* conditions to natural environmental conditions.

The significance of *in vitro* propagation lies in its ability to produce a large number of genetically uniform and disease-free plants within a short period of time. This has immense applications in agriculture and horticulture, particularly for commercially important crops, medicinal plants, and endangered species. Moreover, micropropagation supports year-round plant production, independent of seasonal variations, which enhances its economic value. However, the success of this technique depends heavily on the optimization of culture media, plant growth regulators, light intensity, photoperiod, and temperature conditions.

In addition to callus culture and micropropagation, molecular profiling has become an essential component of modern plant tissue culture systems. Molecular profiling involves the use of DNA-based markers and other molecular techniques to assess the genetic fidelity and diversity of *in vitro* regenerated plants. Techniques such as Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeats (ISSR), Simple Sequence Repeats (SSR), and Amplified Fragment Length Polymorphism (AFLP) are commonly employed for this purpose. These techniques help in detecting genetic variations that may arise during tissue culture and ensure that regenerated plants are genetically identical to the parent material.

The integration of molecular profiling with tissue culture techniques has significantly improved the reliability and efficiency of plant regeneration systems. It allows researchers to monitor genetic stability at various stages of culture and ensures that only true-to-type plants are selected for further multiplication and field transfer. This is particularly important in commercial micropropagation, where uniformity and quality are critical parameters for market acceptance.

Furthermore, advancements in plant tissue culture have also contributed to the fields of genetic engineering, secondary metabolite production, and conservation biology. Callus cultures serve as excellent platforms for genetic transformation studies, where foreign genes can be introduced to develop improved plant varieties with desirable traits such as disease resistance, stress tolerance, and enhanced yield. Similarly, *in vitro* systems are used for the production of valuable secondary metabolites such as alkaloids, flavonoids, and terpenoids, which have pharmaceutical and industrial significance.

Despite its numerous advantages, plant tissue culture also faces several challenges. These include contamination issues, high production costs, genotype dependency, and difficulties in acclimatizing plantlets to natural conditions. Additionally, somaclonal variation remains a major concern, especially in long-term cultures. Therefore, continuous research is required to optimize protocols, improve efficiency, and ensure genetic stability in regenerated plants.

ii. MOLECULAR PROFILING OF IN VITRO REGENERATED PLANTS

Molecular profiling of *in vitro* regenerated plants is a critical component of modern plant biotechnology that ensures the genetic fidelity, stability, and uniformity of plants produced through tissue culture techniques. During *in vitro* regeneration processes such as callus culture, organogenesis, and somatic embryogenesis, plant cells are exposed to artificial culture conditions that may induce genetic or epigenetic variations. These variations, commonly referred to as somaclonal variations, can lead to differences between the regenerated plants and the original donor (mother) plant. Therefore, molecular profiling is essential to confirm that regenerated plants are true-to-type and suitable for further propagation or commercial use.

The primary objective of molecular profiling is to detect and analyze genetic variations at the DNA level. Unlike morphological or physiological observations, which may be influenced by environmental factors, molecular techniques provide highly accurate and reliable information about genetic integrity. This makes molecular profiling an indispensable tool in micropropagation systems, germplasm conservation, genetic transformation studies, and crop improvement programs.

A wide range of DNA-based molecular marker systems are used for profiling in vitro regenerated plants. Among the most commonly used techniques are Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeats (ISSR), Simple Sequence Repeats (SSR), Amplified Fragment Length Polymorphism (AFLP), and more recently, Single Nucleotide Polymorphism (SNP) analysis and next-generation sequencing (NGS)-based approaches. Each of these techniques has unique advantages and limitations in terms of cost, reproducibility, resolution, and technical complexity.

RAPD markers are one of the earliest and simplest methods used for detecting genetic variation. They require no prior sequence information and involve the amplification of random segments of genomic DNA using short primers. Although RAPD is cost-effective and easy to perform, it often suffers from low reproducibility. ISSR markers, on the other hand, target microsatellite regions in the genome and provide higher reproducibility and polymorphism detection compared to RAPD. These markers are widely used for assessing clonal fidelity in micropropagated plants.

SSR markers, also known as microsatellites, are highly polymorphic and co-dominant in nature. They are considered one of the most reliable tools for genetic analysis due to their high reproducibility and specificity. SSR-based profiling is particularly useful for distinguishing closely related plant varieties and detecting subtle genetic differences among regenerated plants. AFLP markers combine restriction enzyme digestion and selective amplification, offering a high level of genomic coverage and sensitivity, making them suitable for detailed genetic stability studies.

In addition to these traditional marker systems, advanced molecular techniques such as SNP genotyping and next-generation sequencing have significantly enhanced the resolution of genetic analysis. SNP markers represent single base-pair changes in the DNA sequence and provide highly precise information about genetic variation. NGS technologies allow comprehensive genome-wide analysis, enabling researchers to identify even minor genetic changes induced during tissue culture processes.

The process of molecular profiling typically begins with the extraction of high-quality genomic DNA from both the donor plant and regenerated plantlets. The DNA is then subjected to polymerase chain reaction (PCR) amplification using selected molecular markers. The resulting DNA fragments are separated using gel electrophoresis or capillary electrophoresis and compared

between samples. Identical banding patterns indicate genetic stability, while differences in banding patterns suggest the presence of somaclonal variation.

Molecular profiling plays a crucial role in ensuring the success of micropropagation systems, especially in commercially important crops and medicinal plants. In large-scale propagation programs, even minor genetic variations can affect yield, quality, and bioactive compound production. Therefore, maintaining genetic uniformity is essential for agricultural and pharmaceutical applications. Additionally, molecular profiling is widely used in germplasm conservation programs to verify the authenticity of preserved plant materials and prevent genetic drift over time.

Another important application of molecular profiling is in plant breeding and genetic improvement programs. Tissue culture techniques are often combined with genetic engineering and mutation breeding to develop improved plant varieties. In such cases, molecular profiling helps confirm the successful integration of desired traits and ensures that no unintended genetic alterations have occurred. This increases the reliability and safety of genetically modified plants before field release.

Despite its advantages, molecular profiling also faces certain limitations. It requires specialized laboratory facilities, skilled personnel, and relatively high operational costs. In some cases, different marker systems may produce inconsistent results, making interpretation complex. However, continuous advancements in molecular biology and bioinformatics are addressing these challenges by improving accuracy, automation, and data analysis capabilities.

iii. INTEGRATION OF CALLUS CULTURE, PROPAGATION, AND MOLECULAR PROFILING

The integration of callus culture, in vitro propagation, and molecular profiling represents a holistic and advanced approach in modern plant biotechnology aimed at achieving efficient plant regeneration, large-scale multiplication, and genetic fidelity assurance. These three components, when combined in a systematic workflow, form a powerful platform for plant improvement, conservation of elite germplasm, and production of uniform, disease-free planting materials. This integrated strategy addresses both the biological and technological challenges associated with plant

tissue culture systems, particularly somaclonal variation, low regeneration efficiency, and genotype dependency.

Callus culture serves as the foundational stage in this integrated system. It involves the induction of an unorganized mass of dedifferentiated plant cells from selected explants under controlled in vitro conditions. The success of callus induction depends on several factors, including the choice of explant, nutrient composition of the culture medium, and the concentration and balance of plant growth regulators. Auxins such as 2,4-D are commonly used to induce callus formation, while cytokinins may be added to influence cell division and morphogenesis. Once established, callus cultures provide a versatile biological system that can be directed toward either organogenesis or somatic embryogenesis, depending on the hormonal and environmental conditions applied. This flexibility makes callus culture a crucial starting point for plant regeneration systems.

Following successful callus induction, the propagation phase begins, which involves the regeneration and multiplication of shoots and roots to produce complete plantlets. In vitro propagation, also known as micropropagation, ensures rapid multiplication of genetically identical plants under sterile conditions. This stage is highly dependent on optimized plant growth regulator combinations, where cytokinins such as BAP promote shoot proliferation and auxins such as IBA or NAA induce root formation. The efficiency of propagation can be further enhanced through careful regulation of environmental factors such as light intensity, photoperiod, temperature, and sucrose concentration in the culture medium. The ultimate goal of this stage is to produce a large number of healthy plantlets that can be transferred to soil conditions after proper acclimatization.

However, one of the major concerns during callus-based regeneration and micropropagation is the possibility of genetic variation arising in cultured tissues. Somaclonal variation, which may occur due to prolonged culture duration, stress conditions, or genetic instability of callus cells, can lead to undesirable changes in the regenerated plants. These variations may affect morphological traits, yield potential, biochemical composition, and stress tolerance. Therefore, ensuring genetic fidelity becomes a critical requirement in any plant tissue culture-based production system.

This is where molecular profiling plays a crucial role in the integrated system. Molecular profiling involves the use of DNA-based marker techniques to assess the genetic stability of regenerated plants in comparison with the original donor plant. Techniques such as RAPD, ISSR, SSR, AFLP,

and SNP analysis are widely used for this purpose. These molecular tools enable precise detection of genetic variation at the DNA level, which cannot be identified through morphological observation alone. By analyzing banding patterns or sequence variations, researchers can determine whether the regenerated plants are genetically identical or have undergone any mutations during in vitro culture.

The integration of molecular profiling into callus culture and propagation systems introduces a quality control mechanism that significantly enhances the reliability of tissue culture protocols. After regeneration, plantlets are routinely screened using molecular markers to confirm clonal fidelity before large-scale multiplication or field transfer. This ensures that only genetically stable and true-to-type plants are selected for further use. As a result, the risk of introducing unwanted genetic variation into commercial plantations or conservation programs is minimized.

The combined workflow of callus induction, plant regeneration, and molecular validation can be summarized as a continuous pipeline: explant selection → callus induction → regeneration via organogenesis or somatic embryogenesis → micropropagation → molecular screening → acclimatization. Each stage is interconnected, and optimization at one stage directly influences the efficiency of the entire system. For example, improved callus induction protocols increase regeneration potential, while efficient micropropagation enhances multiplication rates. Similarly, accurate molecular profiling ensures the final output is genetically stable and commercially viable.

This integrated approach has wide-ranging applications in agriculture, horticulture, forestry, and medicinal plant production. It is particularly valuable for the mass propagation of elite cultivars, endangered species conservation, and production of high-value medicinal plants with consistent phytochemical profiles. In genetic engineering programs, this system is also used to regenerate transformed plants and verify gene integration and expression stability. Furthermore, it supports germplasm conservation efforts by enabling long-term storage and regeneration of genetically stable plant materials.

Despite its advantages, the integrated system also faces challenges such as cost of laboratory setup, requirement for skilled personnel, contamination risks, and occasional inconsistencies in regeneration responses across different plant species. However, advancements in automation,

bioreactor-based culture systems, and high-throughput molecular techniques are continuously improving the efficiency and scalability of these processes.

iv. GENETIC TRANSFORMATION AND GENE EDITING

Genetic transformation and gene editing are two of the most advanced and powerful tools in modern plant biotechnology, enabling precise modification of plant genomes to introduce desirable traits such as disease resistance, abiotic stress tolerance, improved yield, and enhanced nutritional quality. These technologies have significantly expanded the scope of plant breeding beyond conventional hybridization and selection, allowing direct manipulation of genetic material at the molecular level.

Genetic transformation refers to the process of introducing foreign DNA (transgenes) into the genome of a plant cell, resulting in stable or transient expression of new genetic traits. This process typically involves the use of either biological or physical methods for gene delivery. The most widely used biological method is **Agrobacterium tumefaciens-mediated transformation**, where a naturally occurring soil bacterium is engineered to transfer a specific gene of interest into the plant genome. The bacterium contains a Ti (tumor-inducing) plasmid, which is modified by replacing tumor-forming genes with the desired gene sequence. When plant tissues are infected, the T-DNA region of the plasmid integrates into the plant genome, leading to stable genetic transformation.

Another important method is **biolistic particle delivery**, also known as the gene gun method. In this technique, microscopic gold or tungsten particles coated with DNA are physically bombarded into plant tissues. This method is particularly useful for plant species that are less susceptible to *Agrobacterium* infection, such as cereals and monocots. Other less common methods include electroporation, microinjection, and PEG-mediated transformation of protoplasts.

The success of genetic transformation depends heavily on the availability of a reliable plant regeneration system, which is often achieved through callus culture or somatic embryogenesis. After transformation, selection markers such as antibiotic or herbicide resistance genes are used to identify successfully modified cells. These transformed cells are then regenerated into whole plants using optimized tissue culture protocols.

In recent years, gene editing technologies have revolutionized plant biotechnology by enabling highly precise, targeted modifications in the genome without necessarily introducing foreign DNA. The most widely used gene editing tool is the **CRISPR-Cas9 system (Clustered Regularly Interspaced Short Palindromic Repeats associated protein 9)**. This system uses a guide RNA (gRNA) to direct the Cas9 enzyme to a specific DNA sequence, where it introduces a double-strand break. The plant's natural DNA repair mechanisms then repair the break through non-homologous end joining (NHEJ) or homology-directed repair (HDR), leading to gene knockout, insertion, or replacement.

Compared to traditional genetic transformation, CRISPR-based gene editing offers several advantages, including higher precision, lower cost, faster development time, and reduced regulatory concerns in some jurisdictions. It allows researchers to target multiple genes simultaneously (multiplex editing), making it particularly useful for improving complex traits such as drought tolerance, disease resistance, and yield stability.

Other gene editing tools include TALENs (Transcription Activator-Like Effector Nucleases) and ZFNs (Zinc Finger Nucleases), which also enable targeted genome modification but are more complex and less commonly used than CRISPR systems. These technologies have played a significant role in early genome editing studies and continue to have niche applications.

Both genetic transformation and gene editing rely on efficient plant tissue culture systems for regeneration of edited or transformed cells into whole plants. Callus culture is especially important in this context, as it provides a totipotent cell mass that can be easily manipulated and regenerated. After transformation or editing, molecular techniques such as PCR, sequencing, and marker analysis are used to confirm the presence, expression, and stability of the introduced or modified genes.

The applications of these technologies are extensive. In agriculture, genetically modified and gene-edited crops are developed with improved resistance to pests, pathogens, and environmental stresses. In horticulture, traits such as flower color, shelf life, and growth rate can be enhanced. In pharmaceutical biotechnology, plants are engineered to produce vaccines, therapeutic proteins, and valuable secondary metabolites. Additionally, gene editing is increasingly being used in functional genomics studies to understand gene function and regulatory networks.

v. CONCLUSION

Plant tissue culture, combined with advanced molecular and genetic tools, represents a cornerstone of modern plant biotechnology with vast applications in agriculture, horticulture, forestry, and conservation biology. This study highlights the integrated importance of optimized callus culture protocols, efficient in vitro propagation systems, molecular profiling techniques, and modern genetic manipulation approaches such as transformation and gene editing. Together, these methodologies provide a comprehensive framework for rapid plant multiplication, genetic improvement, and quality assurance of regenerated plant materials.

Callus culture serves as the foundational step in plant regeneration systems, offering a totipotent cellular platform for organogenesis and somatic embryogenesis. The optimization of culture conditions, particularly the balanced use of plant growth regulators and controlled environmental factors, significantly enhances regeneration efficiency. However, the occurrence of somaclonal variation remains a critical concern, especially during prolonged culture periods. This challenge necessitates the integration of molecular profiling techniques to ensure genetic fidelity.

In vitro propagation techniques have proven highly effective for the mass production of uniform, disease-free, and high-quality planting materials. The ability to rapidly multiply elite genotypes irrespective of seasonal constraints has revolutionized commercial plant production systems. Nevertheless, the success of micropropagation depends on the standardization of protocols, careful selection of explants, and strict maintenance of aseptic conditions.

Molecular profiling has emerged as an indispensable quality control tool in plant tissue culture systems. The use of DNA-based markers such as RAPD, ISSR, SSR, AFLP, and SNP analysis enables accurate detection of genetic variations and ensures the clonal fidelity of regenerated plants. This step is crucial for maintaining the genetic integrity of propagated materials, particularly in large-scale commercial and conservation programs.

Furthermore, the incorporation of genetic transformation and gene editing technologies has significantly expanded the potential of plant biotechnology. Techniques such as *Agrobacterium*-mediated transformation and CRISPR-Cas9-based genome editing allow precise modification of plant genomes, enabling the development of improved varieties with enhanced agronomic traits.

When combined with efficient regeneration systems, these technologies provide powerful tools for functional genomics and crop improvement.

Overall, the integration of callus culture, in vitro propagation, molecular profiling, and gene editing creates a complete and efficient biotechnological pipeline for plant improvement and conservation. Despite certain challenges such as somaclonal variation, technical complexity, and regulatory constraints, continuous advancements in molecular biology and tissue culture techniques are steadily overcoming these limitations.

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