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Advancements And Challenges In Micropropagation: A Powerful Tool In Modern Plant Biotechnology

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Abstract: Modern biotechnology relies heavily on advancements in plant tissue culture and micropropagation, offering precise control of environmental conditions, efficient plant regeneration, and disease-free mass production within sterile environments. These techniques enable genetic diversity preservation and the cultivation of plants under otherwise inhospitable conditions. Micropropagation involves five stages: explant preparation, aseptic culture initiation, shoot multiplication, rooting of in vitro shoots, and transplantation. Challenges include somaclonal variation, hyperhydricity, and tissue browning, mitigated through analyses, optimal culture conditions, and adsorbents. Despite challenges, micropropagation plays a crucial role in accelerated crop development, disease-free plant production, and genetic conservation, making it an indispensable tool in modern agriculture and horticulture.

***Index Terms*- Micropropagation, Plant tissue culture, Somaclonal variarion, Crop development.**

I. INTRODUCTION

Numerous innovative methods are presently under development, and modern biotechnology heavily relies on the advancement of more efficient procedures for plant tissue culture and micropropagation. These techniques come with a multitude of advantages. Firstly, they allow for precise control of environmental conditions during cultivation, ensuring consistent outcomes regardless of local climate fluctuations. Secondly, they facilitate high rates of plant regeneration, even from tiny tissues or organs, typically measuring less than 1.0 cm in size. Thirdly, they streamline the rapid, efficient, and large-scale production of disease-free plantlets within a sterile environment, significantly reducing the risk of contamination.

Moreover, these techniques facilitate the maintenance of duplicates of vital cultures under in vitro conditions, thus preventing the loss of valuable genotypes. They also allow for the long-term preservation of tissues or organs through cryopreservation, ensuring the conservation of genetic diversity. Lastly, they make it feasible to cultivate plants that would otherwise be unable to survive in uncontrolled environmental conditions, such as haploid plantlets.

Additionally, these methods maintain an aseptic environment, offering superior control over cultivated organisms, whether plants or plant microorganisms, with minimal contamination concerns. They also lend themselves well to automation, enabling the streamlining of various processes and increasing overall efficiency.

Furthermore, plant growth regulators grant precise control over tissue or organ development, making it easier to produce solid mutants or transgenic plants instead of chimeras.

Nevertheless, certain challenges arose in the field of micropropagation during the 21st century. These challenges encompassed enhancing the efficiency of existing micropropagation techniques, limited innovations in the development of new methods, the preservation of recalcitrant species for micropropagation, and the elevated costs associated with micropropagation, potentially leading to the adoption of more economical alternatives.

II. MICROPROPAGATION STAGES

Micropropagation is a well-structured process comprising five distinct stages. These stages include explant preparation, aseptic culture initiation, multiplication, rooting of in vitro shoots, and transplantation. Each stage has its unique set of requirements and challenges, and they collectively enable the efficient mass production of plants from a single explant. This technique plays a pivotal role in the rapid and controlled propagation of plants with desirable traits, making it a valuable tool in agriculture, horticulture, and plant breeding.

2.1 Stage 0- Selection of mother plant

Stage 0 also known as the Preparatory Stage, serves a crucial role in addressing contamination concerns, particularly fungal contamination. However, it is now recognized as essential not only for contamination control but also for the overall success of subsequent stages, namely Stages 1 and 2. This stage encompasses various measures aimed at enhancing the quality of the parent plant, encompassing both hygiene and physiological factors. The cleanliness and health of the mother plants are pivotal for combating contamination issues in Stage 1, where the establishment of aseptic cultures is critical. Typically, explants obtained from plants maintained in a greenhouse environment yield more favorable outcomes. Providing basal irrigation to donor plants minimizes contamination risks during culture initiation, reducing the need for aggressive sterilization measures and increasing the number of healthy cultures. Additionally, Stage 0 may involve adjusting factors like light and temperature conditions in which mother plants are maintained, as well as the application of growth regulators, which can influence explant responses in later stages.

2.2 Stage 1- Culture initiation

Stage 1, commonly referred to as the Aseptic Culture Initiation stage, has a primary goal of establishing plant cultures entirely devoid of contamination. While achieving absolute freedom from infections can be a challenging endeavor, the aim is to consistently attain a high percentage of aseptic cultures, an essential prerequisite for the successful execution of a micropropagation protocol. Key factors influencing success at this stage encompass the careful selection of the appropriate explant, the effective implementation of sterilization techniques, and the prevention of hypersensitivity reactions in the explants.

Explant Selection: The choice of explant hinges on the preferred method of regeneration and multiplication, as well as the specific research objectives. Among the most commonly employed explants for micropropagation are apical buds or nodal segments containing at least one axillary bud. When the goal is virus elimination, meristem tip culture is the method of choice. Nevertheless, for other purposes, meristem tip culture should be avoided due to its low survival rates and intricate culture demands. Opting to propagate from apical and axillary buds offers the advantage of obtaining progeny that are genetically identical to the parent plant.

Sterilization: Conventional sterilization procedures, are typically followed. In cases where field material necessitates micropropagation, it is advisable to first establish donor plants in a greenhouse from cuttings. This precautionary step helps to mitigate contamination risks stemming from wind-borne contaminants. Within a sterile hood, aseptic shoot tips can be meticulously dissected, with outer leaves or scales from buds and bulbs being removed, followed by surface sterilization using 70% ethanol.

Browning:Certain plants, particularly tree species and specific horticultural crops, release phenols from their explants. These phenols can undergo oxidation, resulting in the formation of quinones and a blackening of the culture medium, potentially posing toxicity issues to the tissue. Effective management of this concern is imperative to ensure the health and growth of the cultured plant material.

The successful execution of Stage 1 holds paramount importance as it establishes a clean and uncontaminated foundation for the micropropagation process, setting the stage for subsequent stages with the assurance of a healthy and robust starting point.

2.3 Stage 2- Shoot multiplication

Shoot multiplication is a crucial stage in micropropagation, and the success of the protocol depends largely on its efficiency.

There are three main methods of shoot multiplication:

1. Regeneration from callus: Plant cells from almost any part of a plant can form callus under suitable culture conditions. This callus can then be induced to regenerate plants through organogenesis or somatic embryogenesis. Both methods have the potential to produce a large number of plants, but somatic embryogenesis is much more efficient.
2. Direct adventitious bud formation from the explant: Adventitious buds are buds that form in unusual places, such as on leaves or stems. This method involves culturing an explant (a small piece of plant tissue) under conditions that favor the formation of adventitious buds.
3. Forced axillary branching: This method involves manipulating the plant hormones in the culture medium to promote the growth of axillary buds (buds that form at the junction of a leaf and stem).

Each of these methods has its own advantages and disadvantages. Regeneration from callus is the most versatile method, but it can be slow and inefficient. Direct adventitious bud formation is faster, but it is not possible for all species. Forced axillary branching is the fastest method, but it requires careful control of the culture medium.

The best method of shoot multiplication for a particular species will depend on a number of factors, including the desired number of plants, the availability of explants, and the skill and experience of the grower.
The success of micropropagation protocol is largely dependent on the efficiency of the shoot multiplication stage. Shoot multiplication can be achieved through three methods: regeneration from callus, direct adventitious bud formation from the explant, and forced axillary branching. Each of these methods has its own advantages and disadvantages.

Regeneration from callus is the most common method of shoot multiplication, and it has the potential to produce a large number of plants. However, it can be difficult to synchronize the growth of the callus cultures, and the conversion of embryos into plants can be very poor due to morphological and physiological abnormalities.

Direct adventitious bud formation from the explant is a more efficient method of shoot multiplication, and it is often used for the commercial production of plants. However, it is not suitable for all plants, and the number of shoots per propagule can be limited.

Forced axillary branching is a method of shoot multiplication that involves stimulating the growth of the axillary buds on the explant. This method is often used in conjunction with other methods of shoot multiplication, and it can be very effective in increasing the number of shoots per propagule.

The best method of shoot multiplication for a particular plant will depend on the specific characteristics of the plant and the desired outcome.

2.4 Stage 3- Rooting of shoots

Somatic embryos are complex structures containing both root and shoot primordia, which enables them to develop into complete plants. However, when shoots are generated through various methods like callus regeneration, direct adventitious bud formation, or forced axillary branching, an additional step is required for the formation of roots to create a whole plant. In some cases, when shoots are continuously exposed to cytokinins in vitro, they may remain short and need an intermediate elongation step before transferring them to a rooting medium. This elongation medium can be a liquid medium with the same composition as the one used for shoot multiplication or a medium with reduced levels of cytokinins. It is recommended to transfer shoots in clusters to the selected elongation medium, which not only promotes uniform elongation of all the shoots but also reduces handling costs.

The presence of cytokinins in the medium inhibits root formation, necessitating the transfer of shoots to a suitable rooting medium. Rooting is typically achieved by transferring individual shoots to a medium supplemented with an appropriate auxin. If possible, it is cost-effective to use shoot clusters as a single unit for this step, similar to the approach used for onion and garlic. Rooting is a labor-intensive stage, accounting for about 70% of the cost associated with micropropagated plant production. Therefore, it is crucial to achieve a high rooting success rate (above 95%).

To reduce the cost of rooting micropropagated shoots, many commercial companies employ in vivo rooting. In this method, micropropagated shoots are treated as microcuttings and directly planted in a potting mix after treating the cut basal end with a commercial rooting mix or an auxin solution. In vivo rooting offers several advantages:

1. The roots formed in vitro die after transplantation, and new roots are formed to sustain the plant.

2. The vascular connection between the in vitro formed roots and the shoot may not be well developed.

3. In vitro formed roots lack root hairs, making them less effective when transplanted.

4. Transplantation is typically performed by unskilled labor, and since the plants to be transferred are often quite large, the in vitro roots may get damaged during this process.

5. Callusing at the junction of roots and shoot is a common problem associated with in vitro rooting.

In vivo rooting not only reduces costs but also circumvents the challenges associated with in vitro rooting.

2.5 Stage 4- Transfer of plant in greenhouse conditions

The success of micropropagation ultimately depends on the successful transition of plants from the controlled in vitro environment to soil or a potting mix. In the in vitro setting, plants are exposed to an artificial environment within culture vials. This environment is characterized by a culture medium rich in inorganic and organic nutrients, sucrose, and growth regulators. Additionally, it features high humidity, low light levels, and limited gaseous exchange. Although plants thrive under these unnatural conditions, they exhibit numerous morphological, anatomical, cytological, and physiological abnormalities. These abnormalities necessitate careful acclimatization to adapt the plants to in vivo conditions.

The two primary abnormalities observed in in vitro-grown plants are as follows:

1. Heterotrophic Mode of Nutrition: This arises from the culture medium's richness in organic nutrients, leading to plants heavily relying on external nutrient sources.

2. Poor Control of Water Loss: Due to the high humidity in the culture vials, the leaves of these plants exhibit several issues, including:

- Inadequate development of the cuticle, which is the protective waxy layer on the leaf surface.

- Limited deposition of wax on the leaf surface.

- Abnormally large stomata that fail to close, even in response to ABA (abscisic acid), elevated CO2 levels, or darkness.

- Insufficient differentiation of mesophyll tissue, primarily comprising spongy parenchyma.

- Underdeveloped chloroplasts with low chlorophyll content and disorganized grana.

The absence of a well-developed cuticle and the impaired structure and function of stomata result in excessive water loss upon transplantation, thereby reducing the chances of plant survival. Consequently, it is essential to subject in vitro plants to a careful hardening or acclimatization process before transferring them to field conditions.

2.6 Acclimatization-

The process of acclimatizing in vitro plants, originally accustomed to thriving in environments characterized by high humidity and low light, revolves around transitioning them to conditions with low humidity and abundant light, fostering autotrophic growth. This acclimatization procedure typically spans approximately 4 to 6 weeks to ensure that the plants can flourish under standard conditions.

To begin, individual micropropagated plants are carefully extracted from the agar medium, and their roots undergo a thorough washing to eliminate any residual agar. Subsequently, they are transplanted individually into pots filled with an appropriate potting mix, which may consist of materials such as peat, vermiculite, perlite, polystyrene beads, or coco compost. These plants are irrigated with a mild nutritive solution, such as Knop's solution or a solution composed of one-fourth the strength of MS salt solution.

In research and educational laboratories, a commonly employed and straightforward approach to maintain adequate humidity around the transplanted in vitro plants is to shield them with plastic bags that have small perforations to allow for proper air circulation. Initially, these plants are placed in shaded or low-light conditions for approximately 15 to 20 days. Gradually, they are exposed to lower humidity conditions by periodically removing the polybags for a few hours daily. Over time, the duration of exposure to ambient conditions is incrementally extended until the plants can endure the complete removal of the cover.

Throughout this acclimatization phase, the plants undergo a hardening process, enabling them to adapt to inorganic nutrient sources, while their photosynthetic machinery is reactivated, facilitating their transition to autotrophic growth. For these plants to thrive under field conditions, the development of new roots and leaves is indispensable.

III. GENERAL TECHNIQUES OF MICROPROPAGATION

3.1 Cultures of Axillary and apical bud

The most commonly employed micropropagation technique for large-scale plant production involves the stimulation of axillary shoot multiplication using isolated apical or axillary buds, often in the presence of a relatively high concentration of cytokinin. In this process, the apical or axillary buds typically harbor multiple incipient leaf primordia. The in vitro cultivation conditions are carefully controlled to encourage shoot growth while discouraging the formation of adventitious structures.

3.2 Meristem culture

Meristems represent clusters of undifferentiated cells formed during plant embryogenesis. They maintain a continuous supply of new cells that eventually differentiate into various tissues and organs, forming the fundamental structure of the plant. The technique of shoot meristem culture involves dissecting a dome-shaped portion of the meristematic area from the tip of a selected donor plant's stem and cultivating it on a specialized culture medium. Each dissected meristem consists of the apical dome containing a limited number of the youngest leaf primordia while excluding any developed vascular or provascular tissues. A significant advantage of working with meristems is their high likelihood of being free from pathogenic organisms that may be present in the donor plant, thus ensuring the purity of the cultures. The culture conditions are meticulously controlled to encourage the organized growth of the apex into a shoot, preventing the formation of any unwanted structures and thereby preserving the genetic stability of the regenerated plants.

3.3 Adventitious shoot formation

Adventitious shoot formation is used for micropropagation, genetic transformation, and the study of plant development. In this pathway, new meristems develop spontaneously in vitro, often on stems, roots, or leaf samples, particularly in response to injury or the presence of external growth regulators (a process known as direct organogenesis). Cytokinins are frequently applied to stem, shoot, or leaf cuttings to stimulate the formation of adventitious buds and shoots. These new structures usually emerge close to existing vascular tissues, allowing for their connection with the plant's vascular system to be observed. Sometimes, adventitious organs can also originate from callus tissue forming at the cut surface of explants (referred to as indirect organogenesis). It's important to note that during adventitious shoot regeneration, somaclonal variation, which can have both beneficial and detrimental effects, may occur.

3.4 Somatic Embryogenesis

Somatic embryogenesis is the process where somatic cells give rise to structures that follow a histodifferentiation pattern, resulting in structures resembling zygotic embryos. This phenomenon occurs naturally in certain plant species and can be induced in others through in vitro methods. Somatic embryogenesis can occur directly from cells or organized tissues in explants or indirectly through an intermediate callus stage.

In many plant species, it has been established that the application of the auxins 2,4-D and NAA, at appropriate concentrations, plays a pivotal role in initiating somatic embryogenesis. Additionally, the use of cytokinins like BAP or kinetin can enhance plant regeneration from somatic embryos, especially after the initial induction by auxins in callus or somatic embryos. However, in specific species like, cytokinins alone can induce somatic embryogenesis.

IV. APPLICATIONS

Tissue culture has revolutionized the propagation of commercially valuable plants worldwide, with over a billion plants being produced annually through this method. The ability to multiply plants in vitro from small plant parts has transformed the way we handle plants on a large scale and has found diverse applications.

In vitro-grown plants can be employed for a range of purposes, including mass propagation of selected cultivars or clones throughout the year, the safe transportation of pathogen-free plants, early release of new varieties, and long-term storage. The primary advantage of micropropagation lies in its capacity to initiate cultures from tiny plant samples and rapidly multiply a large number of plants in a short time and within limited space.

Traditionally, it takes approximately 10–15 years for a new cultivar to be developed by a breeder and scaled up for release to farmers. However, micropropagation has the potential to reduce this time by nearly half. Actively growing in vitro plants, being free from pathogens such as bacteria and fungi, can easily pass quarantine inspections, facilitating the rapid introduction of new crops or plants. Given the overexploitation and habitat destruction that have endangered several vital medicinal plants, in vitro multiplication stands as a crucial strategy for conserving these species. In vitro-cultured plants can be stored for extended periods at low temperatures (4°C) and scaled up as needed. Additionally, by adjusting the composition of the growth medium, these cultures can be maintained without subculturing for up to 6 months or longer under controlled conditions.

Tissue culture also plays a pivotal role in maintaining specific sexes in dioecious species for commercial purposes. For instance, in dioecious species like Asparagus officinalis, where the male plants are valuable for their spears, in vitro propagation can be employed to scale up desired male plants for commercial release. Similarly, papaya, another dioecious plant, can have its female plants, which bear fruit, multiplied using in vitro propagation.

V. CHALLENGES OF MICROPROPAGATION

5.1 Somaclonal variation

Micropropagation, presents a challenge due to the potential occurrence of somaclonal variation. This phenomenon involves unexpected genetic or phenotypic changes in regenerated plants. Chromosomal rearrangements play a significant role in generating such variation. Somaclonal variation is not limited to specific plant species but is commonly observed in plants regenerated from callus cultures.

This variation can manifest as changes in genotype or phenotype, with the latter being either of genetic or epigenetic origin. To ensure the genetic uniformity of vegetatively propagated plant material, it is essential to perform cytological, biochemical, and molecular analyses. These analytical techniques offer a rapid and efficient means of detecting undesirable genetic variability compared to traditional methods relying on morphological and physiological assessments.

5.2 Hyperhydricity

Hyperhydricity, also known as vitrification, is a physiological issue that can occur in plant tissue cultures. This condition is characterized by the formation of translucent and water-filled structures. While it generally doesn't pose major problems during the early stages of plant development, it can become more problematic as the plants grow. Hyperhydricity is typically caused by several factors, including an excessive concentration of cytokinins, high water retention within tightly closed containers, or a low concentration of gelling agents in the culture medium.

5.3 Browning of media

In some cases, when plant explants are placed on a culture medium, they release dark-colored compounds (such as phenols and pigments) from their cut ends into the medium. This can lead to tissue and medium browning, which is often associated with poor culture establishment and reduced regeneration capacity. To mitigate this issue, it's important to minimize the damage to explants during isolation and surface disinfection. There are several approaches to prevent tissue browning. One method involves washing the explants in sterile water for 2-3 hours to remove these compounds. Another approach is to frequently subculture the explants onto new medium,

removing any browned tissues in the process. Additionally, you can initiate the culture in a liquid medium and then transfer it to a semi-solid medium. Alternatively, using a porous substrate like paper bridges or adding activated charcoal (AC) or polyvinylpyrrolidone (PVP) to the culture medium can help adsorb these compounds. However, it's important to note that AC may also adsorb growth regulators or be toxic to certain tissues.

The use of antioxidants, such as ascorbic acid, citric acid, L-cysteine, or mercaptoethanol, can also effectively prevent tissue browning in the culture. Excessive browning can pose significant challenges at various stages of shoot regeneration.

VI. CONCLUSION

In conclusion, micropropagation is a powerful biotechnological technique that offers numerous advantages in plant propagation and conservation. It allows for precise control of environmental conditions, rapid and large-scale production of disease-free plants, the maintenance of valuable genotypes, and the preservation of genetic diversity through cryopreservation. Additionally, micropropagation maintains an aseptic environment, is amenable to automation, and offers precise control over tissue or organ development.

The process of micropropagation comprises several distinct stages, including explant preparation, aseptic culture initiation, shoot multiplication, rooting of in vitro shoots, and transplantation. Each stage has its unique requirements and challenges but collectively enables the efficient mass production of plants from a single explant.

Despite its many benefits, micropropagation is not without challenges. Somaclonal variation can lead to unexpected genetic or phenotypic changes in regenerated plants. Hyperhydricity and tissue browning are physiological issues that can affect culture success and plant health. These challenges require careful monitoring and management throughout the micropropagation process.

Overall, micropropagation has revolutionized the field of plant propagation and plays a crucial role in agriculture, horticulture, and plant breeding by providing a means to rapidly and efficiently produce large numbers of plants with desirable traits while preserving genetic diversity and conserving valuable genotypes.

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