In-Vitro Breeding Approaches: Revolutionizing Vegetable Crop Improvement

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In vitro cultivation involves growing plant cells, tissues, and organs in vessels with artificial media, allowing them to divide and regenerate into callus or specific plant organs. In vitro breeding encompasses various techniques such as genetic engineering, mutation breeding, molecular breeding, and plant tissue culture. These methods enable the development of plants with desirable traits by manipulating plant genetics and cellular processes. Plant tissue culture plays a crucial role in supporting these breeding strategies by providing a controlled environment for plant regeneration. Together, these approaches offer advanced solutions for improving plant breeding and crop production.

History of plant tissue culture

- In vitro techniques were developed initially to demonstrate the totipotency of plant cells predicted by Haberlandt in 1902.
- Most of the modern tissue culture media have been derived from the work of Skoog and co-workers during 1950s and 1960s.
- First embryo culture was done by Hanning in 1904 who cultured mature embryos of crucifers.
- In 1960, Cocking produced large quantities of protoplasts by using cell wall degrading enzymes.
- Haploid plants from pollen grains were first produced by Maheshwari and Guha in 1964 by culturing anthers of Datura.

Plant tissue culture

Plant tissue culture involves several key stages, starting with the selection and maintenance of a healthy mother plant, followed by the preparation of nutrient media. The sterilization of the nutrient medium, explant, and other items is critical to maintaining aseptic conditions, which are essential for successful tissue culture. After sterilization, the explant is inoculated onto the nutrient medium under sterile conditions. The plant material is then grown in a controlled growth room, sub-cultured as necessary to encourage shoot proliferation, and finally hardened to acclimate micro-plants for transfer to external environments.

Sterilization is a crucial step in tissue culture to eliminate microorganisms and ensure aseptic conditions. Different sterilization methods are employed depending on the type of material. Steam sterilization, commonly performed using an autoclave, involves the use of superheated steam under high pressure (121°C, 15 *psi*, for 20 minutes) to sterilize media, water, and small instruments. Dry sterilization, performed in a hot air oven, uses dry heat at 160–180°C for three hours to sterilize glassware and metallic instruments but is less efficient due to slower heat penetration.

For heat-sensitive materials, filter sterilization is preferred, utilizing membranes with pore sizes of 0.22–0.45 µm to remove microbes. UV sterilization is occasionally used for disposable plasticware, requiring 15–20 minutes of exposure, though it is less common due to higher costs. By combining these methods, sterile media, containers, and instruments can be prepared, ensuring the success of tissue culture procedures.

Concentration and time of exposure of various sterilizing agents

Sterilizing agent	Concentration %	Time of exposure (min)
Sodium hypochlorite (NaOCl)	0.1 - 3	1-20
Calcium hypochlorite ($Ca(ClO)_2$)	1 - 5	5 - 30
Sodium dicholoro isocyanurate (DICA)	1 - 2	10 - 20
Mercuric (II) chloride ($HgCl_2$)	0.1 - 1	2 - 10
Silver nitrate ($AgNO_3$)	1	5 - 20
Hydrogen peroxide ($H2O_2$)	10 - 30	5 - 15

Types Of In Vitro Culture

- Seed Culture
- Organ Culture
- Protoplast Culture
- Bud Culture
- Meristem Culture
- Callus Culture
- Cell Culture
- Embryo Culture

1. Seed Culture

Seed culture involves the in vitro cultivation of seeds on artificial media to produce seedlings or plants. This technique enhances germination efficiency, especially for seeds that are difficult to germinate or show poor germination rates in vivo. It is also employed to raise sterile or aseptic seedlings for further use in research or propagation. Seed culture is particularly beneficial for plants like orchids, tomato, chilli, and capsicum, which may require precise environmental conditions for successful germination.

2. Meristem Culture

Meristem culture, also known as Meri stemming or Meri cloning, involves the in vitro cultivation of apical meristems, particularly shoot meristems, on artificial media. This method is primarily used for plant propagation and the production of virus-free planting materials. By culturing the meristem, which is usually free of systemic pathogens, healthy and disease-free plants can be developed. Meristem culture is widely applied to crops like potato, chilli, capsicum, tomato, and brinjal for commercial production and research purposes.

3. Bud Culture

Single Node Culture (SNC) and Axillary Bud Culture are widely used techniques for in vitro plant propagation. In Single Node Culture, a nodal segment is isolated from the third or fourth node from the stem apex, and the bud is encouraged to develop into a shoot on nutrient media. This method is simple, efficient, and commonly employed for propagating various plants in vitro. Axillary Bud Culture involves isolating an axillary shoot bud and growing it under high cytokinin concentrations. The cytokinin suppresses apical dominance, promoting the development of axillary buds into shoots.

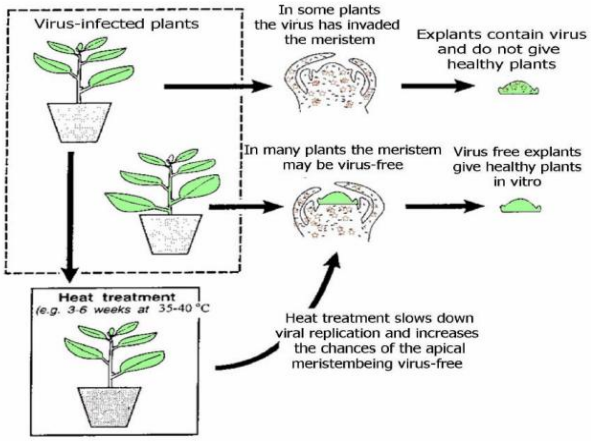

Fig 1: Production of virus free plants through meristem culture

These methods offer several advantages, including being simple and quick, enabling direct organogenesis without callus formation, and achieving high multiplication rates while maintaining genotypic uniformity among propagated plants. This makes them ideal for commercial and research applications, especially in crops like potato, tomato, chili, and capsicum, which benefit from their efficiency and reliability in producing uniform plants.

4. Callus Culture

Callus is an undifferentiated, tumour-like mass of cells. Callus culture involves the aseptic in vitro cultivation of this tissue on artificial media. Regeneration through callus culture includes two key processes: **de-differentiation**, where quiescent cells of the explant revert to a meristematic state to form callus, and **re-differentiation**, where the callus differentiates into shoots and roots, ultimately regenerating into a complete plant. This method is widely used for crops such as potato, tomato, chili, capsicum, and brinjal.

Phases Of Callus Growth

1. Lag Phase – where cells prepare to divide.
2. Exponential Phase – where the rate of cell division is highest.
3. Linear Phase – where cell division slows but the rate of cell expansion increases.
4. Deceleration Phase – where the rate of cell division and elongation decreases.
5. Stationary Phase – where the no. and size of cells remains constant

5. Cell Culture

Cell Suspension Culture involves the growth of single cells or cell aggregates in liquid media, commonly referred to as suspension culture. It is initiated by transferring explant pieces or friable calluses into a liquid medium, which is continuously agitated on a rotary shaker to ensure proper aeration and cell dispersion. This method supports rapid cell division and growth, making it effective for large-scale propagation.

There are two main types of suspension cultures: Batch Culture, where cells grow in a fixed volume of medium until nutrient depletion halts growth and Continuous Culture where fresh medium is added periodically to maintain constant volume and nutrient supply. Applications include large-scale clonal propagation through embryogenic suspensions, germplasm storage and secondary metabolite production. Notable examples include capsaicin production in *Capsicum frutescens*, diosgenin in *Dioscorea* spp., and 3-N-butyl-phthalide in celery, which is effective against hypertension.

6. Organ Culture

Two in vitro methods have been used;

1. Culture of excised ovaries and ovules - OVULE CULTURE
2. Culture of excised anthers and pollens - ANTHER CULTURE

Ease of Anther Culture

a) Ovule is very difficult to separate from the complex tissue integration.
b) Rate of survival is very less in case of ovule culture.
c) Only 1 plantlet can be obtained from single ovule

Another culture is the aseptic excision and cultivation of developing anthers from unopened flower buds on a nutrient medium, where pollen grains are induced to form callus or embryoids, eventually developing into haploid plantlets. The process by which haploid plants arise from male gametophytes is called androgenesis. The procedure involves collecting and sterilizing flower buds, excising and separating anthers, crushing them in 1% acetocarmine, and inoculating them on a nutrient medium. Cultures are maintained under alternating light (12–18 hours) and darkness (6–12 hours) at 28°C. Anthers proliferate, producing callus or embryos, leading to haploid plants that can be converted to double haploids using colchicine treatment.

Types of Androgenesis

1. **Indirect Androgenesis**: Anthers produce callus, which undergoes organogenesis to form plantlets. E.g., asparagus, beetroot.
2. **Direct Androgenesis**: Embryos emerge directly from pollen walls, bypassing callus formation, resulting in plantlets. E.g., tomato, chilli, potato.

Double Haploidy: Haploid plants fail to form viable gametes due to the absence of homologous chromosomes. Chromosome duplication (diploidization) is achieved using colchicine (0.4%) to produce homozygous diploids with minimal gene instability, accelerating variety development and fixing heterosis.

Somatic Embryogenesis: Somatic embryogenesis involves the development of somatic cells into differentiated embryos, which can form plantlets without sexual fertilization. Initiation occurs directly from explants or indirectly via callus formation. Key types include:

1. **Direct SE**: Explant → Somatic Embryo → Plantlets.
2. **Indirect SE**: Explant → Callus → SE → Plantlets.
3. **Recurrent SE**: Explant → Callus → Primary SE → Secondary SE → Plantlets. This process was first observed in *Daucus carota* (Reinert, 1958; Steward *et al*., 1958).

Synthetic Seeds: Synthetic seeds are artificially encapsulated somatic embryos, shoot buds, or meristematic tissues that retain the ability to grow into plants under in vitro and ex vitro conditions, even after storage (Capuano *et al*., 1998; Ara *et al*., 2000).

8. Embryo Culture

Embryo culture involves isolating immature or mature embryos under aseptic conditions and cultivating them on nutrient media. This technique is widely used for embryo rescue in F1 hybrids derived from distant hybridization to overcome pre- and post-fertilization barriers. It also aids in propagating seeds with short viability or minimal endosperm and in shortening breeding cycles. Examples include legumes such as green gram, black gram, French bean,

soybean, and vegetables like tomato, brinjal, potato, and turnip. The first embryo culture was conducted by Hanning (1904) using *Cochlearia* and *Raphanus*.

Embryo rescue has achieved significant milestones in vegetable breeding. For instance, hybrids of *S. lycopersicum* with *S. peruvianum* have been developed for Tospovirus tolerance (Sohrab *et al*., 2014) and resistance to Fusarium wilt and early blight (Kharkongar *et al*., 2013). Similarly, *Capsicum annuum* crosses with *C. chinense* and *C. frutescens* have led to anthracnose resistance, drought tolerance, and resistance to cucumber mosaic virus (Debbarama *et al*., 2013). In cucurbits, *Cucumis metuliferus* × *C. anguria* hybrids were developed for root-knot nematode resistance (Fassuliotis and Nelson, 1998). These examples highlight the utility of embryo rescue in overcoming barriers to hybridization and enhancing crop resilience.

9. Somatic hybridization

Also known as parasexual hybridization or protoplast fusion, this technique eliminates gametes in the hybridization process. It involves fusing the protoplasts (naked plant cells without cell walls) of two different species, genera, or families under in vitro conditions to form a hybrid product called a heterokaryon. Protoplasts are produced by treating plasmolysis cells with a mixture of cellulose and pectinase enzymes. The culture medium for protoplasts resembles that used in plant tissue culture but contains no ammonium and has a higher calcium concentration.

Cybrids are hybrids that contain the nucleus from one parent and the cytoplasm from both parents, achieved through a process called cybridization, where the nucleus of one protoplast is inactivated using agents like iodoacetate. This technique has been used to transfer traits like herbicide resistance and cytoplasmic male sterility (CMS) in crops such as tobacco and tomato.

Methods of Protoplast Fusion

- **Polyethylene Glycol Method:** Protoplasts are suspended in a polyethylene glycol solution, briefly shaken, left undisturbed, and then washed and resuspended in the culture medium.
- **Sodium Nitrate Treatment:** Protoplasts are incubated in sucrose solution, treated with sodium nitrate, and centrifuged before transferring to a suitable culture medium.
- **Electrofusion:** A short electrical shock induces fusion between protoplasts.

Applications of Somatic Hybridization

- Enables interspecific and intergeneric crosses between sexually incompatible plants.
- Produces fertile diploids and polyploids from sexually sterile plants.
- Facilitates the development of unique hybrid plants and transgenic plants.

Micropropagation Applications

Micropropagation is an efficient method for mass multiplication of elite clones, producing plants rapidly from small vegetative parts. It allows year-round plant production in controlled environments, produces disease-free plants, and is ideal for conserving rare or endangered species. Additionally, it enables embryo rescue to save hybrids from incompatible crosses and allows for the establishment of gene banks. Plants produced through this method often exhibit increased vigor, branching, and flowering.

Disadvantages of Micropropagation

Challenges include contamination by slow-growing microorganisms, the accumulation of inhibitory phenolic compounds that darken the medium, and water-soaked leaves in in vitro shoots, which reduce growth and survival rates. Tissue-cultured plantlets often face low survival rates during the hardening phase.

Conclusion

Plant tissue culture is one of the most promising applications in biotechnology today, with significant potential for future advancements. Globally, the biotech industry is valued at around 50 billion, with plant tissue culture products contributing approximately 10% of this figure. The commercialization of plant tissue culture, coupled with the global acceptance of tissue culture-derived plantlets by commercial nursery traders, has driven the industry's growth. Additionally, in vitro screening plays a crucial role in isolating new and improved cell lines, enabling the regeneration of plants with enhanced traits.

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