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Review Article

A Review on Micropropagation Culture Method

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A B S T R A C T

Micropropagation is a vegetative propagation conducted under controlled and aseptic conditions in the microenvironment of the culture vessel, which have the all growth requirements of a plant in the natural conditions. Recently different techniques of propagation have been developed which could facilitate large scale production of plants and for the improvement of the species. An overview on the in vitro propagation via meristem culture, callus culture and protoplast culture etc. are presented here. Today micropropagation techniques are applied in order to produce large numbers of new high-quality plants in a relatively short time and space, in low cost and can also be preserved.

Key words: Micropropagation, meristem culture, totipotency

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INTRODUCTION

The term Micropropagation is generally used for the aseptic culture of cells, tissues, organs and their components under defined chemical and physical conditions in-vitro.[1]

The basic concept of the plant body can be dissected into smaller part termed as "explants "and any explants can be developed into a whole plant.

The plant tissue culture medium is an artificial nutrient supplement of organic and inorganic nutrients used for cultivation of plant tissue media. The culture media used for the in vitro cultivation of the plant cells are composed of three basic components- Essential elements (normal ions), an organic supplements and a source of fixed carbon. When cultured in an appropriate medium having auxin and cytokinin, explants gives rise to an unorganized, growing and dividing mass of cells called "callus".

The applications of plant tissue culture can be divided into following areas; Cell behaviour, plant modification, germ plasm storage and pathogen –free plants, clonal propagation and product form.

The success of micro propagation involves several factors, as the composition of the culture medium, culture environment, genotype etc. [2]

Principles of Micropropagation.[3]

Plant micro propagation is an integrated process in which cells, tissues or organs of selected plants are isolated, sterilized, and incubated in a growth-promoting aseptic environment to produce many clone plantlets. The technique of cloning isolated *demonstrated* the fact that somatic cells, under appropriated conditions, can differentiate to a whole plant.

This potential of a cell to grow and develop a multicellular organism is termed cellular totipotency. [4]

This potential of cells or tissues to form all cell types and regenerate a plant is the basic principle of Micropropagation.[5]

Historical development [6]

S.No.	Name of scientist	year	work
1.	Gottlieb Haberlandt	1902	Develop the concept of in vitro cell culture. He was the first to culture isolated, fully differentiated cells in a nutrient medium.
2.	Kotte and Robbins	1922	Small excised root tips of pea and maize cultivated by kotte. Robbin- Maintained his maize root tip culture for a longer period by sub culturing them.
3.	White	1934 1937	Rowin root tip culture. Initially used yeast extract in a medium containin inorganic salts and sucrose but later replaced yeast extract by three vitamins (pyridoxine, thiamine and nicotinic acid)
4.	Bonner	1937	Importance of thiamine in yeast extract.
5.	Laibach	1925 1929	They raised zygotic embroys isolated from non viable seed of Linum perenne X L austriacum to maturity on a culture medium and obtained hybrid, which in natural course died out due to embryo abortion
6	Van overbeek	1941	Used coconut milk [embryo sac fluid] for embryo development and callus formation in Datura, which proved turnin point in the field of embroyonic culture
7	Ball	1945	Identify the exact part of the shoot meristem that give rise to a whole plant
8	Gauthret	1934	Cultured cambium cell of some tree specis [Acer pseudoplantanus salix caprea] on the surface of medium solidified with agar
9	Caplin steward	1948	Carrot explant that coconut milk enhances more proliferation of callus than did auxin
10	Skoog Skoog and Tsui	1944,1951	Adenine stimulate cell division and induces bud formation in tobacco tissue even in the presence of indoleacetic acid
11	Skoog and Miller	1957	The concept of hormonal control of organ formation from callus tissue
12	Sanford	1948	Initiated study of single cell cultures by demonstrating division in animal cell using conditional media
13	Steward	1952	Designed shaking apparatus, which allowed dissociation of tissue to form cell suspension culture
14	Jones	1960	Designed a micro culture method using hanging drop free cell in a conditioned medium
15	Hildebrandt	1965	Substituted fresh medium enriched with coconut milk and I-Napthalene acetic acid for conditioning and observe division in isolated tobacco hybrid cell
16	Bergmann	1960	Developed cloned from a large no of cells
17	Kohienbah	1966	Isolated mesophyll cells of McCleayer cordata differentiated somatic embroys
18	Ball	1946	Successfully raised transplantable whole plant of Lupinus and Tropaeolum by culturing their shoot meristem
19	Morel	1963	Rapid propagation of Orchid cymbidium and odontoglossum
20	Morel and Martin	1952,1955	Recovered virus free dahlia and potato plant from culture obtained by cultivating the shoot meristem of infected plants culture
21	Guha and Maheshwari	1966	Clyure immature anther of Datura innoxia and were able to raise embroiyds and plantlets
22	Bourgin Nitsch	1967	The titopotency of pollen grain by rising full haploid plant of tobacco, wheat and rice
23	San Noeum	1976	In vitro culture of ovary isolated from Hordeum vulare
24	Bajaj	1976	Regenerated plant from cryopreserved plant tissue
25	Murashieg	1977	Proposed artificial seed production
26	Melcher	1978	Production of somatic embryo's by using protoplast fusion technique
27	Lazer	1983	Production of hybrids by protoplast fusion technique

General method

Preparation of nutrient medium: A semi-solid medium is prepared in double distilled water containing macro elements, micro elements, amino acids, vitamins, iron source, carbon source like sucrose and phyto-hormones.

Establishment of aseptic culture: The starting material for the process is normally an actively growing shoot tip of axillary or terminal bud or shoot tip of a plant.

Inoculation: Inoculation is carried out under aseptic conditions. In this process explants or micro shoots are transferred on to the sterilized nutrient medium.

Development of plant in growth room: After the inoculation of the plant tissue, the bottles are sealed and transfer red into growth room to trigger developmental process under diffused light and humidity.

Hardening of micro plants: Due to very high humidity inside the culture vessel and artificial conditions of development, the plantlets retender and are therefore are not ready for coping up with the field conditions.

Procedure-

Root Tip Culture Method[7, 8, 9]

- Wipe down and turn on the laminar air flow 15 minute before doing work in the hood. Flames sterilize the instruments.
- Cut the root into 3-6 cm long, discarding both ends of the root. Remove the epidermis and any blemishes with scalpel.
- Put the tap root section in to a sterile jar having chlorate bleach solution (approximately 1.4% available chlorine) and shake it for few seconds.
- Remove the bleach solution into the waste beaker.
- Cut 1cm of the root section from each end and discard the end portions.
- Cut 3-5 transverse section (1-5mm thick) across the tap root and transfer each to a fresh sterile Petri dish.
- Cut the smaller sections, explants (approximately 5mm square) from each of the transverse sections by
- cutting across the cambium.
- Measure the weight of the explants.
- Put each explants sectioninto culture tubes containing the callus initiation medium (one explants per tube).
- Seal all the tubes with paraffin to reduce dehydration of the medium.
- Incubate the culture tubes in the dark at 25°C. Examine at weekly intervals and record the changes
- observed.
- Callus formed is removed from the primary explants after 45 days and it is weighed.
- The callus is subculture into the same medium for further callus growth or to the shoot / root initiation
- medium.
- Seal all the tubes having callus with paraffin to reduce dehydration of the medium.
- Incubate all the tubes in the dark at 25 °C.
- Measuring the efficacy of roots and shoots.

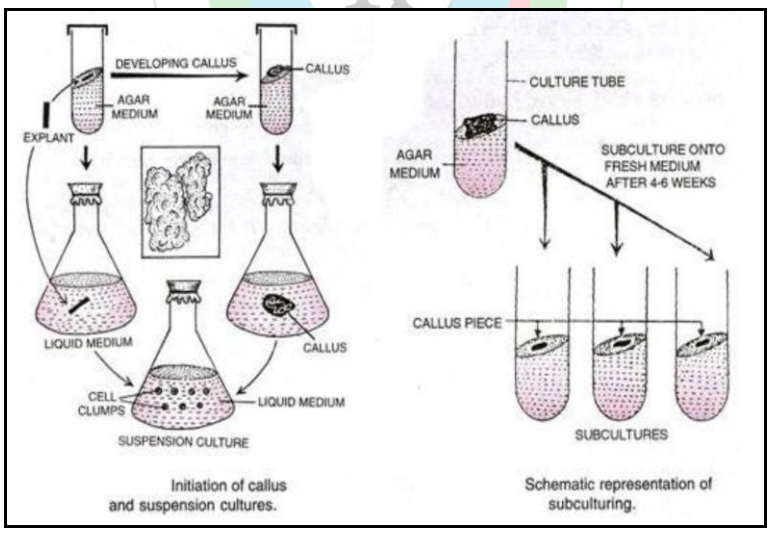

Figure 1: Diagrammatic representation of micro propagation technique

Types of MicropropagationTechniques[10]

- Apical meristem culture
- Axillary bud culture
- Callus culture
- Cell and organ culture
- Suspension culture
- Protoplast culture
- Embryo culture
- Shoot& Root -tip culture
- Flower organ culture
- Fruit organ culture
- Microspore and anther culture

Table 1: Different Culture Techniques with their Advantage and Disadvantages

S.No.	Type of culture	Properties	Advantage	Disadvantage
1	Apical meristem culture	Cultivation of apical meristem in vitro is known as meristem culture.	1.Virus elimination	1.isolation is difficult
		It involves the growth of an already existing shoot meristem and regeneration of adventitious roots from these shoots. [11,12]	2. produces a Large number of plants in a short span of time.	2.Low survival rate
2	Protoplast culture	Protoplast culture consist of decomposition of cell wall of a plant cell and removal by the treatment of the lytic enzymes like cellulose and pectinase, thus contain all the normal cell organelles as well as nucleus.	1.No binary vector is required.	1. Yields a very small amount of protoplasm
			2.High frequency transformation	2.Not suitable for isolating protoplast from
			3. delivery of multiple plasmids	meristematic and less vacuolated cell
3	Callus culture	Callus tissue means an unorganized proliferative mass of cells produced from isolated plant cell, tissue or organ when grown aseptically on artificial nutrient medium under controlled experimental conditions and this form of culture is called as callus culture.	1. Helps in the production of secondary plant products.	1.Specialized Equipment is required
			2. starting materials for vegetative propagation of plants	2. High labour cost
4	Suspension culture	Suspension culture is a type of culture in which single cells or small aggregates of cells multiply while suspended in agitated liquid medium. [13]	1.High Productivity	1.Contamination risk
			2.Better Economics	2.Equipment failure
5	Microspore and anther c culture	Pollen grains from pollen tube and male gametes, when placed on suitable nutrient media, most pollen grain follow normal development, but a few grains will form a callus.	1.Simple	1.Often fail to grow in vitro
			2. Less time consuming	2.Not economically viable for haploid
			3. Responsive	production.

Table 2: Various Plant using Culture Media

S. No.	Plant	Culture
1.	Taxus wallichiana Taxus canadensis	Callus , Cell suspension,
2.	Nothapodytes foetida	Callus , Cell suspension,
3.	Coleus forskohlii	hairy root
4.	Hypericum perforatum Catharanthus roseus	shoot cultures
5.	Catharanthus roseus	hairy

Factors affecting in Vitro Propagation

Media:-

Significant effect of media has been observed on plant regeneration from different parts of plant. [14]

Various basal media like White medium, Nitsch and Nitsch medium, B5 medium and Gamborg medium for micropropagation. [15] [16][17] have been employed, but most widely used culture medium is MS media [18]because most of the plants respond favorably to MS medium, since it contains all the nutrients essential for plant growth in vitro.

Mineral Nutrition:-

Minerals are important components of the culture medium. There is a large choice of combinations of macro -and micro-salt mixtures. The most widely used culturemedium is described in Murashige and Skoog (1962) (MS medium),

because most plants react to it favorably. It contains all the elements that have been shown to be essential for plant growth in vitro.

Carbon Source

Sucrose is by-far the most used carbon source, for several reasons. It is cheap, readily available, relatively stable to autoclaving, and readily assimilated by plants. Other carbohydrates can be also used, such as glucose, maltose and galactose as well as the sugar-alcohols glycerol and sorbitol [19] The carbohydrates added to the culture medium supply energy for the metabolism

Growth Regulators

Growth regulators are organic compounds naturally synthesized in higher plants, which influence growth and development. There are several classes of plant growth regulators, as e.g. cytokinins, auxins, gibberellins, ethylene and abscisic acid.

Gelling Agents

Culture media can be classified as liquid or solid. The liquid media have the advantage of faster (and cheaper) preparation than the solid ones. Agar [20]has traditionally been used as the preferred gelling agent for tissue culture, and is very widely employed for the preparation of semi-solid culture media. [21, 22, 23]

PHYSICAL ENVIRONMENT

Gaseous

The response of plant tissue culture in vitro can besignificantly affected by the gaseous constituents in andadjacent to the culture vessel. Carbon dioxide, oxygenand ethylene are the most frequently studied constituentsof the culture atmosphere.[24] It is generally accepted that the relative humidity in the vessel is approximately 98-100%.[25]

Light

Light is an important environmental factor that controls plant growth and development, since it is related to photosynthesis, phototropism and morphogenesis. The three features of light, which influence in vitro growth, are wavelength, flux density and the duration of light exposure or photoperiod

Temperature

Temperature influences the various physiological processes, such as respiration and photosynthesis, is well known and it is not surprising that it profoundly influences plant tissue culture and micropropagation. The most common culture temperature range has been between 20°C - 27°C, but optimal temperatures vary widely, depending on genotype. [26, 27, 28]

Table 3: Description of Morphogenesis Response[29]

A	Anther culture
A-C	Callus from anthers
C	Callus formed/callus culture
C-IE	Embryonic callus
C-IR Indirect adventitious root formation from callus	Indirect adventitious root formation from callus
C-I Indirect adventitious shoot formation from callus	indirect adventitious shoot formation from callus
C-ISR	Indirect adventitious root and shoot formation from callus
C-R	Roots formed on callus
DE	Direct embryogenesis
DR	Direct adventitious root formation
DR-pre	Root initiation pre-treatment
DRS	Direct root and shoot formation
DS	Direct adventitious shoot formation
E-PI	Plants developed from embryos
G	In vitro germination
GEN	Genetic transformations
IE	Indirect embryogenesis
O-R	Isolated root culture
O-R-S	Root Culture and Shoot Formation
PP	Protoplast culture
Pp-c	Callus formation from protoplasts
Pp-co	Protoplast cultivation
Pp-gen	Protoplast culture for genetic information
Ppi	Protoplast isolation
Pre	Preconditioning

S	Shoot growth (no propagation)
S -DR	Shoot development and direct adventitious root formation
S -Gen	Regenertion of transformed plants
SN	Nodal culture
SN-P	Multiple shoots from single node culture
ST	Shoot tip culture
ST-P	Multiple shoots from shoot tip culture
ST-Pre	Shoot Preconditioning
SU	Suspension culture

Problem and Remedy [30, 31, 32,33,34,35]

Selection of Explant

Explant selection is the initial step in the micropropagation, Any part of the plant can be selected as explants. It should be from healthy mother plant.

Sterilization of media and culture vessel

It is the most important step in tissue and organ culture techniques is sterilization. It is controlled by chemical and physical methods, dry heat, wet heat, chemical and ultra-filtration.

Explant Sterilization

Surface sterilization of the explants is the essential step in the micropropagation. If explants taken from the external environment was exposed to microbial contamination will leads to the mortality of the plant tissue. Microbial contamination is the external part of the explants, can be sterilization with the running water and chemical substance, includes ethanol, sodium hypochlorite, mercuric chloride, and plant preservative mixtures etc.

Effect of ultra violet-c radiation in explant sterilization

The explants are allowed to ultra violet -c radiation for 5 min and surface sterilization for about 10 min, act as a better way to reduce the external contamination. The intensity of UV C- Radiation can deactivate the DNA in the pathogen.

Roll of Endophylactic organism in contamination

Epiphylactic microbes in the explants were removed by surface sterilization but the occurance of endophylactic microbes in the explants were leads to contamination in the culture.

Media preparation, Nutrients and Harmonal Imbalance

Media is the place where explants are inoculated to grow and the success of tissue culture depends upon the selection of media. Most commonly used tissue culture is MS by murashing and scrooge.

The nutritional media consist of inorganic salt, organic supplement, Vitamin, growth regulators and carbon source. Agar is used as gelling agent in the culture medium.

Right pH is required for the medium and optimum pH of the nutrient medium is maintained properly (5.6-5.8).

Plant growth hormones are naturally synthesized by the plants but there is a need to add some external growth harmones for better growth and enrich the metabolite synthesis.

Plant growth hormones are auxin, cytokines, gibberellin. [36]

Contamination

Contamination is the comman problem in the in- vitro culture. Endophytic microbial contamination was not possible to stop with the surface sterilization. Aseptic condition in the invitro culture is the best way to eradicate contamination.

Construction of tissue laboratory

The well designed and planned laboratory with washing room, sterile room, growth culture room, store room and hardening area is quite necessary for successful culture. There should be a well-designed and proper construction of various rooms, which may have less chance of contamination.

Cryopreservation of Cultured Plants[37]

Cryopreservation is a storage method of plant genetic resources at ultra-low temperature, for example, that of liquid nitrogen (-196 °C). It is apreservation method that enables plant genetic resources to be conserved safely, and cost-effectively. For successful cryopreservation, it is essential to avoid intracellular freezing and induce the vitrification state of plant cells during cooling.

Different cryopreservation procedures have been developed- 1. Slow-prefreezing method 2. Vitrification method 3. Dehydration method

Low Cost Approaches [38,39]

Micro propagation technology is more expensive than the conventional methods of plant propagation, and requires several types of skills.

Now this problem has been solved by inventing reliable and cost-effective tissue culture methods without compromising on quality.The cost of the medium per liter was worked out with modifications in its components [40]

Examples-

- Alternatives to Carbon source
- Alternatives to Agar
- Alternatives to distilled water

Table 4: Cost of Conventional and Low-Cost Alternative Source

Modified Conventional	Low Cost	Cost in 1L of the medium (KShs)		Cost reduction(%)
MS medium (CM)	Substitute(LCM)	Conventional	Low Cost	
	Macronutrients			
CaCl2	-	1.6	-	0
KH2PO4	Monopotassium phosphate (MKP)	0.6	0.04	93.2
KNO3	Potassium fertilizer	6.8	0.34	94.9
MgSO4	Epsom Salt	1	0.07	93
NH4NO3	Ammonium quary salt	10	0.38	96.2
Sub- total		20	2.43	87.8
Micronutrients				
CoCl2.6H2O		0.005		
CuSO4.5H20		0.005		
Na2EDTA		0.15		
FeSO4.7H2O		0.08		
H3BO3	Stanes iodized microfood	0.1		
KI		0.07		
MnSO4.4H2O		0.09		
Na2MoO4.2H2O		0.008		
ZnSO4.7H2O		0.02		
Sub-total		0.538	0.24	28.9
Carban source				
Sucrose	Table sugar	105	3	97.1
Total		125.538	5.67	g

Advantages of Micropropagation[41]

- The micropropagation method produces plants free of diseases.
- Large amounts of plants can be maintained in small spaces. This helps to save endangered species and the storage of germplasm.
- The micropropagation technique is also useful for seed production in certain crops as the requirement of genetic conservation to a high degree is important for seed production.
- This method holds better scope and future for the production of important plant-based phytopharmaceuticals.
- Micropropagation offers a lucrative alternative approach to conventional methods in producing controlled amounts of biochemical.

Limitations of Micropropagation

Expensive laboratory equipment and service
Plants are not autotrophic
Poor Acclimatization to the field is a common problem
Risk of genetic changes if 'de novo' regeneration is used

Applications of Micropropagation

- Somaclonal Variation
- Germplasm Conservation
- Mutation Breeding
- Inducing mutation
- Embryo culture
- Haploid and Diploid production.[42]
- In Vitro Hybridization- protoplast fusion
- Production of Disease-free plants
- Molecular farming
- Genetic engineering
- Production of secondary metabolites
- Rapid growth and Bioassay
- The presence of the petiole inhibits the outer growth of lateral buds, which were already present in the inoculated bud. By usingmicropropagation, the growth potential of auxiliary buds can be enhanced.
- The process can also be used to develop the plants that are resistant to various kinds of stresses. [43]

CONCLUSION

The micropropagation technique is useful for large-scale plant multiplication through cutting down the cost of production per plant by applying low-cost tissue culture, and

plant production without compromising the quality. It is rapid multiplication process of a selected plant. Micropropagation result in true to type plant.

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