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Review Article

Micropropagation as an advanced technology for the conservation of plants

Seema Tiwari[1] and Rashmi Arnold[2]

1, Dept. of Biotechnology, Govt. T.R.S. College, Rewa (M.P.), India
2, Dept. of Botany Govt. T.R.S. College, Rewa (M.P.), India

Abstract

Plant tissue culture techniques are essential to many types of academic inquiry, as well as to many applied aspects of plant science. In the past, plant tissue culture techniques have been used in academic investigations of totipotency and the roles of hormones in cytodifferentiation and organogenesis. Currently, tissue-cultured plants that have been genetically engineered provide insight into plant molecular biology and gene regulation. Plant tissue culture techniques are also central to innovative areas of applied plant science, including plant biotechnology and agriculture. For example, selected plants can be cloned and cultured as suspended cells from which plant products can be harvested. In addition, the management of genetically engineered cells to form transgenic whole plants requires tissue culture procedures; tissue culture methods are also required in the formation of somatic haploid embryos from which homozygous plants can be generated. Thus, tissue culture techniques have been, and still are, prominent in academic and applied plant science.

Keywords: Micropropogation, Plants, Tissue culture

Introduction

The term micropropagation means a technological process consisting of several steps in order to produce numerous propagules from a chosen mother plant under *in vitro* conditions. Large scale technologies have been developed following long experimental procedures worldwide. In the period of 1970-1980 research was focused onto the methodology of sterile culturing, and the propagation of economically important plant species. Trials with different nutrient media, investigation on growth regulator effects, experiments concerning interaction of genotype and environmental effects – some of the most important topics at this period. From the beginning of the nineties attention was payed to new technologies, scale-up and automatization. It can be stated that in this field of science theory and practice forms a strong unity, both in improving the knowledge and in developing new technologies.

Micropropagation is used routinely to generate a large number of high-quality clonal plants, including medicinal, agricultural, ornamental and vegetable species, and in some cases also plantation crops, fruits and vegetable species. Micropropagation has significant advantages over traditional clonal propagation techniques. These include the potential of combining rapid large-scale propagation of new genotypes, the use of small amounts of original germplasm and the generation of pathogen-free propagules[1].

One of the most exciting and important aspects of *in vitro* cell and tissue culture approach is the capability to regenerate and propagate plants from cultured cells and tissues. Micropropagation involves the production of plants from very small plant parts, tissues, or cells grown aseptically in test tubes or other containers where the environment and nutrition can be rigidly controlled[2]. The inherent capacity of a plant cell to give rise to a whole plant, a capacity which is often retained even after a cell has undergone final differentiation in the plant body is described as "cellular totipotency[3]. For a differentiated cell to express its totipotency it first undergoes dedifferentiation followed by redifferentiation. Tissue culture techniques offer not only an excellent opportunity to study the factors that elicit the totipotentiality of cells but also allow investigation of factors controlling cytological, histological and organogenetic differentiation[4].

***Corresponding Author**

E.mail - seemat452@gmail.com
Mob. +919201428721

Process of Micropropagation

The process of plant micro-propagation aims to produce clones (true copies of plant in large numbers). The process is usually divided into the following stages:

Stage 0- pre-propagation step or selection and pre-treatment of suitable plants.
Stage I- initiation of explants-surface sterilization, establishment of mother explants.
Stage II- subculture for multiplication/ proliferation of explants.
Stage III- shooting and rooting of explants.
Stage IV- weaning/ hardening.

These stages are universally applicable in large-scale multiplication of plants. The individual plant species, varieties and clones require specific modification of the growth media, weaning and hardening conditions. A rule of the thumb is to propagate plants under conditions as natural or similar to those in which the plants will be ultimately grown *ex-vitro*. For example, if a chrysanthemum variety is to grown under long day-length for flower production, it is better to multiply the material under long day-length at stages III and IV. There is a wide option to undertake production of plant material up to a limited number of stages. For example, many commercial tissue culture companies undertake production up to stage III, and leave the remaining stages to others[5]..

This impressive application of the principles of plant cell division and regeneration to practical plant propagation is the result of continuous tedious studies in hundreds of laboratories worldwide, many of them in developing countries, on the standardization of explant sources, media composition and physical state, environmental conditions and acclimatization of *in vitro* plants. Particularly noteworthy are the many recent studies on the molecular of organogenesis and somatic embryogenesis. However, further practical applications of micropropagation, which is also commercially viable, depends on reducing the production costs such that it can compete with seed production or traditional vegetative propagation methods (e.g., cuttings, tubers and bulbs, grafting). Indian tissue culture laboratories are among the pioneering ones in establishing technologies for *in vitro* multiplication of several species[6].

The application of micropropagation techniques provides several benefits viz.: -

It enhances the rate of rapid multiplication of plants, producing thousands of plantlets in a matter of months.
It provides the availability of plants throughout the year.
It helps the conservation of genetic resources of threatened plants.
Plant improvement can be brought about by regeneration techniques Healthy plant material is ensured since soil and disease-causing organisms are excluded during the propagation cycle.
The method is programmable to meet specific targets of time and quantity because it is independent of seasonal changes and the weather.
Micropropagation saves an enormous amount of care usually required by cuttings and seedlings (watering, weeding, spraying etc.)
Excess material produced can often be stored over long periods.
Species and cultivars can be stored in small spaces[7].

Need of Micropropagation Technique In Plants:

- Rarely found in nature while doing micropropagation it grows easily everywhere.
- It can make thousands identical copies of mother plant with in short time.
- To improve quality & quantity of plant.
- To make disease free plant.
- To make economical beneficial.
- From *in vitro* propagation we can provide all the favorable conditions during growth of plants.

Plant tissue culture techniques have a vast potential to produce plants of superior quality, but this potential has been not been fully exploited in the developing countries. During *in vitro* growth, plants can be primed for optimal performance after transfer to soil. In most cases, tissue-cultured plants out-perform those propagated conventionally. Thus in *vitro* culture has a unique role in sustainable and competitive agriculture and forestry, and has been successfully applied in plant breeding, and for the rapid introduction of improved plants. Bringing new improved varieties to market can take several years if the multiplication rate is slow. For example, it may take a lily breeder 15-20 years to produce sufficient numbers of bulbs of a newly bred cultivar before it can be marketed. *In vitro* propagation can considerably speed up this process. Plant tissue culture has also become an integral part of plant breeding. For example, the development of pest and disease- resistant plants through biotechnology depends on a tissue culture based genetic transformation. The improved resistance to disease and pests enables growers to reduce or eliminate the application of chemicals.
The FAO Committee on Agriculture has perceived plant tissue culture as a main technology for the developing countries for the production of disease-free, high quality planting material, and its commercial application in floriculture and forestry [8]. It further points out that tissue culture techniques are being used particularly for large-scale plant multiplication. Micropropagation has proved especially useful in producing high quality, disease-free planting material for a wide range of crops. Tissue culture based industry also generates much-needed rural employment, particularly for women.
The broad application of existing technologies to plantation species is important for tree improvement in the tropics[9]. In a small number of plantation programs, micropropagation is being used as an early rapid multiplication step. However, it has been pointed out that the current high costs will be an impediment to the direct use of micropropagules as planting stock. Micropropagation clearly has a role, in the rapid multiplication of the selected clones for conventional production of cutting. The direct use of micropropagules as planting stock in industrial plantation can dramatically broaden forestry tree farming if propagation costs are reduced.
The availability of micropropagation technologies will also useful in genetic engineering applications, e.g., the production of plant as a source of "edible" vaccines. There are many other useful plant-derived substances which can be produced in tissue cultures, sometimes more cheaply and reliably than from natural forests and plantation. These include medicinal compounds and drugs now being sought in major prospecting operations in the tropical forests.
Micropropagation has been identified as a technology in the development projects of UNESCO in Africa and the Caribbean; however, the cost of production must be reduced[10]. Practically in all developing countries, the private industry is the most important group that requires cost-effective technology. For example, in India of the 90 commercial micropropagation units established initially, 32 were closed down. Of those engaged in commercial production, many are uneconomic mainly due to the high cost of culture production[11]. And the absence of quality tests, hence, low-cost tissue culture technology will stay a high priority in agriculture, horticulture, forestry, and floriculture of many developing countries.

Future Role in Plant Tissue Culture Techniques

It has been stressed time and again that in the long- term agriculture and forestry need to be sustainable, use little or no crop-protection chemicals, have low energy inputs and yet maintain high yields, while producing high quality material. Biotechnology-assisted plant breeding is an essential step to achieve these goals. Plant tissue culture techniques have a vast potential to produce plants of superior quality, but this potential has been not been fully exploited in the developing countries. During *in vitro* growth, plants can be primed for optimal performance after transfer to soil. In most cases, tissue-cultured plants out-perform those propagated conventionally. Thus in *vitro* culture has a unique role in sustainable and competitive agriculture and forestry, and has been successfully applied in plant breeding, and for the rapid introduction of improved plants. Bringing new improved varieties to market can take several years if the multiplication rate is slow. For example, it may take a lily breeder 15-20 years to produce sufficient numbers of bulbs of a newly bred cultivar before it can be marketed. *In vitro* propagation can considerably speed up this process. Plant tissue culture has also become an integral part of plant breeding. For example, the development of pest and disease- resistant plants through biotechnology depends on a tissue culture based genetic transformation. The improved resistance to disease and pests enables growers to reduce or eliminate the application of chemicals.
The FAO Committee on Agriculture has perceived plant tissue culture as a main technology for the developing countries for the production of disease-free, high quality planting material, and its commercial application in floriculture and forestry [8]. It further points out that tissue culture techniques are being used particularly for large-scale plant multiplication. Micropropagation has proved especially useful in producing high quality, disease-free planting material for a wide range of crops. Tissue culture based industry also generates much-needed rural employment, particularly for women.
In forestry, the availability of tissue culture linked production systems may effectively provide sustainable alternatives to the

need for harvesting wood from native forests and natural habitats. Successful protocols now exist for the micropropagation of a large number of forest tree species, and the number of species for which successful use of somatic embryogenesis is increasing. Thus in the future, it is likely that, micropropagation in the forestry sector will become commercially important. Compared to vegetative propagation through cuttings, the high multiplication rates available through Micropropagation offers a much quicker capture of genetic gains obtained in forest tree breeding programs. However, the current high costs will also be one of the major impediments to the direct use of micropropagation in many programs.

The broad application of existing technologies to plantation species is important for tree improvement in the tropics[9]. In a small number of plantation programs, micropropagation is being used as an early rapid multiplication step. However, it has been pointed out that the current high costs will be an impediment to the direct use of micropropagules as planting stock. Micropropagation clearly has a role, in the rapid multiplication of the selected clones for conventional production of cutting. The direct use of micropropagules as planting stock in industrial plantation can dramatically broaden forestry tree farming if propagation costs are reduced.

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Explants Collections

Leaf, nodal and inter-nodal segments may be collected as explants from several days (2-4 months) old plant for micropropagation.

Surface Sterilization Of Explants

Surface sterilization is necessary in order to disinfect the explant before it was placed over media. Following steps are:The explant were placed in different bottles and covered with net and washed for 30 minutes under running tap water to remove all the dust particles and microbes from the surface. In the next step explants were soaked in an aqueous soap solution containing 1% Labolene (Qualigens) for 5-7 minutes and then washed with distilled water. Explants were treated with 2% Bavistin (antifungal) for 10 minutes and antibiotic for 5 minutes. This was followed by gentle wash in double distilled water for 5 minutes for two cycles.

Sterilization Procedure Under Aseptic Conditions

All the experimental manipulations were carried under aseptic conditions in an inoculation chamber fitted with a bactericidal ultraviolet tube (15W, peak emission 2537A^{o}). The floor of the chamber was thoroughly scrubbed with cotton dipped in alcohol. The surface of all the vessels and other accessories such as instruments (spatula, forceps, needles coupling jar and scalpel etc.), spirit lamp, matchbox, sterile double distilled water, tube containing absolute alcohol etc. were also cleaned with alcohol. The fresh material to be inoculated was kept in a Petri dish covered with a piece of filter paper in order to protect it from the harmful effects of ultraviolet rays. Alcohol was then sprayed in the chamber with the help of an atomizer. The chamber was then sterilized with ultraviolet tube kept continuously on for one hour.

After the surface sterilization explants were taken inside the laminar flow hood. Here 2-3 washings were given with sterile double distilled water. Further explants were surface disinfected with freshly prepared 0.1% (w/v) aqueous solution of mercuric chloride for 5 minutes. They were then thoroughly washed for 3-4 times with sterile double distilled water to remove any traces of mercuric chloride for 5 minutes (two cycles). The explants were immersed in 70% ethanol for 5 min. This was followed by at least three rinses in double distilled water. Rinsed explants were inoculated in prepared Murashige and Skoog's medium (MSM) (Murashige and Skoog., 1962).

Initiation of Cultures

Sterilized explants were transferred aseptically to sterilized glass plate under the laminar flow hood. Then a cut was given on both basal as well as the top portion of the explants to remove undesirable/dead portions after surface sterilization. The forceps were earlier rinsed in the 70% ethanol (Bengal Chemicals and Pharmaceuticals Ltd.) and were flamed and then kept for sometime to get cool. Then the lid from one test tube was removed and test tube's mouth was flamed to avoid any chance of contamination. Each nodal explant was then placed in an erect position in the test tube containing medium with the help of long forceps. The lid was finally closed carefully, flamed lightly and sealed with kiln film. The forceps were then again rinsed with 70% alcohol to avoid any chance of cross contamination. The same procedure was undertaken for all the explants. These jars were finally kept in the growth room with temperature conditions 25± 2 ^{o}C, with a photoperiod of 16 hours daylight and 8 hrs night break under the cool white fluorescent light of average 2500 lux (cool white fluorescent tube light 40 W GE).

Glasswares

The glasswares used for culture work comprised of 6"Õ 1" borosil test tubes, 250ml, 500ml and 1000ml borosil flasks. In addition other glassware includes graduated measuring cylinder, Petri dishes, beakers and a range of pipettes. Before use, glasswares were thoroughly brushed with alkaline detergent tepol and then washed in running tap water. It was then treated with hot chromic acid (mixture of $K_2Cr_2O_7$ + H_2SO_4 + H_2O) followed by very thorough washing with tap water. All vessels were then inverted in a clean tray and left to dry. Copper distilled water (5-10ml) was then poured into every culture vessel which was tightly plugged. Plugs were made out of absorbent surgical cotton wrapped in muslin. Glassware was then steam sterilized in an autoclave at a pressure of 15 lb\in2 (121^{0}C) for 15 to 20 minutes.

Culture Medium

Plant Tissue Culture refers to the technique of growing plant cells, tissues, organs, seeds or other plant parts in a sterile environment on a nutrient medium. Culture media used for *in vitro* cultivation of plant cells are composed of following basic components:

Complex mixture of salts: Essential elements, or mineral ions., Organic supplement: vitamins and/or amino acids, Carbon source: usually sugar sucrose, Gelling agents, Plant Growth Regulators, Antibiotics

The composition of the nutrient medium is an important aspect of successful plant regeneration schedule. *In vitro* studies are carried on Murashige and Skoog's medium (MSM) (Murashige and Skoog., 1962).

Plant growth regulators

Plant growth regulators play important role in the development and growth of plant. They are usually active at different site and present in very small quantities.

Various concentrations of different auxins viz. 2,4-D (2,4-Dicholorophenoxy acetic acid) (0.5-5 mg/L), NAA (Naphthalene acetic acid) (0.5-5 mg/L), IAA (Indole-3 acetic acid) (0.5-5 mg/L) and IBA (Indole butyric acid) (0.5-5 mg/L) and Cytokinins viz. BAP (6-Benzyl amino purine) (0.5-5 mg/L) and Kinetin (KN) (6-Furfuryl amino purine) (0.5-5 mg/L) were used.

Preparations Of Medium

Nutritional requirement for optimal growth of a tissue in vitro may vary with the species. As such, no single medium can be suggested as being entirely satisfactory for all types of plant tissues and organs. When starting with a new system it is essential to work out a medium that would fulfill the requirement of that tissue. In order to formulate a suitable medium for a new system it would be better to start with a well known medium such as MS. By making minor changes, through a series of experiments, a new medium may be evolved to accommodate specific requirements of the plant material in question. The concentrated stocks of the major salts, minor salts and growth regulators were prepared and stored under refrigeration. Auxins were normally prepared by dissolving in 1N KOH and cytokinins in 1 N HCl before making up the final volume with distilled water.

Auxins are generally used in plant cell culture at a concentration range of 0.01-10.0 mg/l. When added in appropriate concentrations they may regulate cell elongation, tissue swelling, cell division, formation of adventitious roots inhibition of adventitious and axillary shoot formation, callus initiation and growth, and induction of embryogenesis.

Cytokinins are generally used in plant cell culture at a concentration range of 0.1-10.0 mg/l. When added in appropriate concentrations they may regulate cell division, stimulate auxiliary and adventitious shoot proliferation, regulate differentiation, inhibit root formation, activate RNA synthesis, and stimulate protein and enzyme activity.

For the routine media preparation, the necessary volume of each stock solution in each medium was pipetted out into the conical flasks. The required amounts of plant growth regulators were added. Sucrose crystals were dissolved by addition of double distilled water. The volume was raised to a little less than the final volume. The pH of the media was adjusted to 5.6-5.8 by adding 1N HCl or 1N NaOH drop wise. Then each medium was made up to the required volume. Prior to the addition of agar-agar, the medium was heated to help quick dissolution of the same. The medium was then dispensed into clean glass culture tubes (Borosil; 18 X 150 mm) and the liquid medium into conical flasks (Borosil; 150, 250 ml) and enclosed with plugs made of non-absorbent cotton wrapped in muslin cloth. Culture tubes were grouped into bunches and covered with aluminium foil. Media were autoclaved at a pressure of 15 psi and a temperature of 121ºC for 15 minutes. Autoclaved media was left at 25ºC for 24 hrs to check that there was no visible microbial contamination.

Culture Conditions

The cultures were maintained in culture tubes and conical flasks and were kept in the culture room at a temperature of 25±2ºC, relative humidity (RH) of 60-70% and a light intensity of approx. 2500 lux provided by cool, white, fluorescent tubes under a photoperiod of 16/8 hr (light/dark).

Subcultures

Cultures were maintained through regular monthly subcultures. The cultured tissues were aseptically transferred on to fresh media without being subjected to chemical sterilization. To achieve shoot elongation, the multiple shoot clusters were dissected into finer units. The adhering agar and necrotic tissues were removed without damaging the shoot primordial/buds. These units were finally recultured on to fresh media having appropriate plant growth regulators (PGRs). Subcultures were also performed as and when necessary after evaluation of growth changes.

Conclusion

Finally we can conclude that Plant tissue culture is used widely in plant science; it also has a number of commercial applications like Micropropagation is widely used in forestry and in floriculture. It can be used to conserve rare or endangered plant species. A plant breeder may use tissue culture to screen cells rather than plants for advantageous characters, e.g. herbicide resistance/tolerance. Large-scale growth of plant cells in liquid culture in bioreactors for production of valuable compounds, like plant-derived secondary metabolites and recombinant proteins used as biopharmaceuticals. Micropropagation using meristem and shoot culture to produce large numbers of identical individuals. So it is working as an advance technology for the conservation of plants.

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