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Conservation of Biodiversity Through Tissue culture

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India.**Keywords:** micropropagation,
protoplasts, synthetic seed, haploids,
secondary products**ABSTRACT**

Biodiversity is the degree of variation of life forms within a given ecosystem, biome or an entire planet. Biodiversity is a measure of the health of ecosystems. Biodiversity is in part a function of climate. Plant tissue culture comprises a set of *in vitro* techniques, methods and strategies that are part of the group of technologies called plant biotechnology. Tissue culture has been exploited to create genetic variability from which crop plants can be improved, to improve the state of health of the planted material and to increase the number of desirable germ plasms available to the plant breeder. Tissue-culture protocols are available for most crop species, although continued optimization is still required for many crops, especially cereals and woody plants. Tissue culture techniques, in combination with molecular techniques, have been successfully used to incorporate specific traits through gene transfer. In vitro techniques for the culture of protoplasts, anthers, microspores, ovules and embryos have been used to create new genetic variation in the breeding lines, often via haploid production. Cell culture has also produced somaclonal and gametoclonal variants with crop-improvement potential. The culture of single cells and meristems can be effectively used to eradicate pathogens from planting material and thereby dramatically improve the yield of established cultivars. Large-scale micropropagation laboratories are providing millions of plants for the commercial ornamental market and the agricultural, clonally-propagated crop market. With selected laboratory material typically taking one or two decades to reach the commercial market through plant breeding, this technology can be expected to have an ever increasing impact on crop improvement as we approach the new millenium.

INTRODUCTION

Biodiversity is the vast array of all the species of plants, animals, insects and microorganisms inhabiting the earth either in the aquatic or the terrestrial habitats. Global community has experienced major changes as a result of the unfolding and globalization of revolution taking place since years. Ecological degradation and its corollary – biodiversity loss – pose a serious threat to development. 'Ecologically destructive economic activities are inefficient not merely because of the resulting resource misallocation but also because of the (excessive) scale of activity levels; excessive in relation to the limited availability of natural capital when the latter is complementary to human-made capital'. In order to bring about sustainable resource conservation and management, it is essential to adopt several different approaches for managing our forests and biodiversity. Plant tissue culture forms an integral part of any plant biotechnology activity. It offers an alternative to conventional vegetative propagation.

Tissue culture has opened exciting frontier in the field of agriculture and offers opportunities for the increase in productivity, profitability, stability and sustainability^[13,14]. Plant tissue culture techniques have also helped in large-scale production of plants through micropropagation or clonal propagation of plant species. Small amounts of tissue can be used to raise hundreds or thousands of plants in a continuous process. This is being utilized by industries in India for commercial production of mainly ornamental plants like orchids and fruit trees, e.g., banana. The idea of culturing isolated cell was given by Haberlandt^[3]. Using this method, millions of genetically identical plants can be obtained from a single bud. This method has, therefore, become an alternative to vegetative propagation. Shoot tip propagation is exploited intensively in horticulture and the nurseries for rapid clonal propagation of many dicots, monocots and gymnosperms.

Clonal propagation refers to the process of asexual reproduction by multiplication of genetically identical copies of individual plants. The vegetative propagation of plants is labour-intensive, low in productivity and seasonal. The tissue culture methods of plant propagation, known as 'micropropagation' utilizes the culture of apical shoots, axillary buds and meristems on suitable nutrient medium. The regeneration of plantlets in cultured tissue was described by Murashige in 1974. The micropropagation is rapid and has been adopted for commercialization of important plants such as banana, apple, pears, strawberry, cardamom, many ornamentals (e.g. Orchids) and other plants [7].

The micro propagation techniques are preferred over the conventional asexual propagation methods because of the following reasons:

- (a) In the micropropagation method, only a small amount of tissue is required to regenerate millions of clonal plants in a year.,
- (b) Micro propagation is also used as a method to develop resistance in many species.,
- (c) In vitro stock can be quickly proliferated as it is season independent.,
- (d) Long term storage of valuable germplasm possible.

The steps in micropropagation method are:

- a) Initiation of culture – from an explant like shoot tip on a suitable nutrient medium,
- b) Multiple shoots formation from the cultured explant,
- c) Rooting of in vitro developed shoots and,
- d) Transplantation – transplantation to the field following acclimatization.

The factors that affect micropropagation are:

- (a) Genotype and the physiological status of the plant e.g. plants with vigorous germination are more suitable for micropropagation.,
- (b) The culture medium and the culture environment like light, temperature etc. For example an illumination of 16 hours a day and 8 hours night is satisfactory for shoot proliferation and a temperature of 25°C is optimal for the growth.

The benefits of micropropagation these methods are:

- a) Rapid multiplication of superior clones can be carried out throughout the year, irrespective of seasonal variations.
- b) Multiplication of disease free plants e.g. virus free plants of sweet potato (*Ipomea batatas*), cassava (*Manihot esculenta*)
- c) Multiplication of sexually derived sterile hybrids
- d) It is a cost effective process as it requires minimum growing space.

Production of virus free plants

The viral diseases in plants transfer easily and lower the quality and yield of the plants. It is very difficult to treat and cure the virus infected plants therefore the plant breeders are always interested in developing and growing virus free plants. In some crops like ornamental plants, it has become possible to produce virus free plants through tissue culture at the commercial level. This is done by regenerating plants from cultured tissues derived from a) virus free plants, b) meristems which are generally free of infection – In the elimination of the virus, the size of the meristem used in cultures play a very critical role because most of the viruses exist by establishing a gradient in plant tissues. The regeneration of virus-free plants through cultures is inversely proportional to the size of the meristem used., c) meristems treated with heat shock (34–36°C) to inactivate the virus, d) callus, which is usually virus free like meristems.e) chemical treatment of the media– attempts have been made to eradicate the viruses from infected plants by treating the culture medium with chemicals e.g. addition of cytokinins suppressed the multiplication of certain viruses. Among the culture techniques, meristem-tip culture is the most reliable method for virus and other pathogen elimination [8].

Somaclonal variation

The genetic variations found in the in vitro cultured cells are collectively referred to as somaclonal variation and the plants derived from such cells are called as 'somaclones'. It has been observed that the long-term callus and cell suspension culture and plants regenerated from such cultures are often associated with chromosomal variations. It is this property of cultured cells that finds potential application in the crop improvement and in the production of mutants and variants (e.g. disease resistance in potato). Somaclonal variation is one of the potential aspects of tissue culture which is widely used for crop improvement specially for obtaining desired traits in salt, drought, frost, temperature and disease resistance [1].

Embryo Culture

Hannig [1904] initiated a new line of investigation involving the culture of embryogenic tissue. He excised nearly mature embryos of some Crucifers and successfully grew them to maturity on mineral salts and sugar solution. Van Overbeek et al [15] used coconut milk (embryo sacfluid) for embryo development and callus formation in *Datura* which proved a turning point in the field of embryo culture. A new approach to tissue culture was conceived simultaneously by Kotte (Germany) and Robbins (USA) [4] in 1922. They postulated that a true in vitro culture could be made easier by using meristematic cells, such as those that operate in the root tip or bud. An important breakthrough for continuously growing root tip cultures came from White (1934,1937), who initially used yeast extract in a medium containing inorganic salts and sucrose but later replaced it by three B vitamins, namely, pyridoxine, thiamine and nicotinic acid. White's synthetic medium later proved to be one of the basic media for a variety of cell and tissue cultures.

Production of synthetic seeds

In synthetic seeds, the somatic embryos are encapsulated in a suitable matrix (e.g. sodium alginate), along with substances like mycorrhizae, insecticides, fungicides and herbicides. These artificial seeds can be utilized for the rapid and mass propagation of desired plant species as well as hybrid varieties. The major benefits of synthetic seeds are:

They can be stored up to a year without loss of viability.

- a) Easy to handle and useful as units of delivery
- b) Can be directly sown in the soil like natural seeds and do not need acclimatization in green house.

Mutantselection

An important use of cell cultures is in mutant selection in relation to crop improvement. The frequency of mutations can be increased several fold through mutagenic treatments and millions of cells can be screened. A large number of reports are available where mutants have been selected at cellular level. The cells are often selected directly by adding the toxic substance against which resistance is sought in the mutant cells. Using this method, cell lines resistant to amino acid analogues, antibiotics, herbicides, fungal toxins etc have actually been isolated.

Production of Secondary Metabolites

The most important chemicals produced using cell culture are secondary metabolites, which are defined as 'those cell constituents which are not essential for survival'. These secondary metabolites include alkaloids, glycosides (steroids and phenolics), terpenoids, latex, tannins etc. It has been observed that as the cells undergo morphological differentiation and maturation during plant growth, some of the cells specialize to produce secondary metabolites. The in vitro production of secondary metabolites is much higher from differentiated tissues when compared to non-differentiated tissues. Many of these secondary products especially various alkaloids are of immense use in medicine. The yield of these chemicals in cell culture, is though generally lower than in whole plants, it is substantially increased by manipulating physiological and biochemical conditions [10,11]. Shikonine is a dye produced by the cells *Lithospermum erythrorhizon* on a commercial scale. Besides this there are a number of secondary metabolite products that are being widely used for various purposes. Vincristine is used as anticancer agent, digoxin controls cardiovascular disorders, pyrethrin is an insecticide etc. The production of specialty chemicals by plants has become a multibillion industry.

Production of Somatic hybrids and cybrids

The Somatic cell hybridization/ parasexual hybridization or Protoplast fusion offers an alternative method for obtaining distant hybrids with desirable traits significantly between species or genera, which cannot be made to cross by conventional method of sexual hybridization.

Somatic hybridization broadly involves in vitro fusion of isolated protoplasts to form a hybrid cell and its subsequent development to form a hybrid plant. The process involves: a) fusion of protoplasts, (b) Selection of hybrid cells, (c) identification of hybrid plants. During the last two decades, a variety of treatments have been used to bring about the fusion of plant protoplasts. Protoplast fusion can be achieved by spontaneous, mechanical, or induced fusion methods. These treatments include the use of fusogens like NaNO_3 , high pH with high Ca^{2+} ion concentration, use of polyethylene glycol (PEG), and electrofusion. These inducing agents used in protoplast fusion are called 'fusogen'. PEG treatment is the most widely used method for protoplast fusion as it has certain advantages over others. These are : (a) it results in a reproducible high-frequency of heterokaryon formation., (b) The PEG fusion is non specific and therefore can be used for a wide range of plants., (c) It has low toxicity to the cell and (d) The formation of binucleate heterokaryons is low. The methods used for the selection of hybrid cells are biochemical, visual and cytometric methods using fluorescent dyes. The biochemical methods for selection of hybrid cells are based on the use of biochemical compounds in the medium. The drug sensitivity

method is useful for the selection hybrids of two plants species, if one of them is sensitive to a drug. Another method, auxotrophic mutant selection method involves the auxotrophs which are mutants that cannot grow on a minimal medium. Therefore specific compounds are added in the medium. The selection of auxotrophic mutants is possible only if the hybrid cells can grow on a minimal medium. The visual method involves the identification of heterokaryons under the light microscope. In some of the somatic hybridizations, the chloroplast deficient protoplast of one plant species is fused with the green protoplast of another plant species. The heterokaryons obtained are bigger and green in colour while the parental protoplasts are either small or colourless. The cytometric method uses flow cytometry and fluorescent-activated cell sorting techniques for the analysis of plant protoplasts.

Table showing plant species and secondary metabolites obtained from them using tissue culture techniques

Product	Plant source	Uses
Artemisin	<i>Artemisia spp.</i>	Antimalarial
Azadirachtin	<i>Azadirachta indica</i>	Insecticidal
Berberine	<i>Coptis japonica</i>	Antibacterial, anti inflammatory
Capsaicin	<i>Capsicum annum</i>	Cures Rheumatic pain
Codeine	<i>Papaver spp.</i>	Analgesic
Camptothecin	<i>Camptotheca acuminata</i>	Anticancer
Cephalotaxine	<i>Cephalotaxus harringtonia</i>	Antitumour
Digoxin	<i>Digitalis lanata</i>	Cardiac tonic
Pyrethrin	<i>Chrysanthemum cinerariaefolium</i>	Insecticide (for grain storage)
Morphine	<i>Papaver somniferum</i>	Analgesic, sedative
Quinine	<i>Cinchona officinalis</i>	Antimalarial
Taxol	<i>Taxus spp.</i>	Anticarcinogenic
Vincristine	<i>Cathranthus roseus</i>	Anticarcinogenic
Scopolamine	<i>Datura stramonium</i>	Antihypertensive

Cybrids

The cytoplasmic hybrids where the nucleus is derived from only one parent and the cytoplasm is derived from both the parents are referred to as cybrids. The process of formation of cybrids is called cybridization. During the process of cybridization and heterokaryon formation, the nuclei are stimulated to segregate so that one protoplast contributes to the cytoplasm while the other contributes nucleus alone. The irradiation with gamma rays and X-rays and use of metabolic inhibitors makes the protoplasts inactive and non-dividing. Some of the genetic traits in certain plants are cytoplasmically controlled. This includes certain types of male sterility, resistance to certain antibiotics and herbicides. Therefore cybrids are important for the transfer of cytoplasmic male sterility (CMS), antibiotic and herbicide resistance in agriculturally useful plants. Cybrids of Brassica raphanus that contain nucleus of B. napus, chloroplasts of atrazinc resistant B. capestris and male sterility from Raphanus sativas have been developed.

In vitro plant germplasm conservation

Germplasm refers to the sum total of all the genes present in a crop and its related species.

The conservation of germplasm involves the preservation of the genetic diversity of a particular plant or genetic stock for its use at any time in future. It is important to conserve the endangered plants or else some of the valuable genetic traits present in the existing and primitive plants will be lost. A global organization- International Board of Plant Genetic Resources (IBPGR) has been established for germplasm conservation and provides necessary support for collection, conservation and utilization of plant geneic resources throughout the world.

The germplasm is preserved by the following two ways:

(a) In-situ conservation- The germplasm is conserved in natural environment by establishing biosphere reserves such as national parks, sanctuaries. This is used in the preservation of land plants in a near natural habitat along with several wild types.

(b) Ex-situ conservation- This method is used for the preservation of germplasm obtained from cultivated and wild plant materials. The genetic material in the form of seeds or in vitro cultures are preserved and stored as gene banks for long term use.

In vivo gene banks have been made to preserve the genetic resources by conventional methods e.g. seeds, vegetative propagules, etc. In vitro gene banks have been made to preserve the genetic resources by non - conventional methods such as cell and tissue culture methods. This will ensure the availability of valuable germplasm to breeder to develop new and improved varieties. The methods involved in the in vitro conservation of germplasm are:

(a) Cryopreservation- In cryopreservation (Greek-krayos-frost), the cells are preserved in the frozen state. The germplasm is stored at a very low temperature using solid carbon dioxide (at -79°C), using low temperature deep freezers (at -80°C), using vapour nitrogen (at- 150°C) and liquid nitrogen (at-196°C). The cells stay in completely inactive state and thus can be conserved for long periods. Any tissue from a plant can be used for cryopreservation e.g. meristems, embryos, endosperms, ovules, seeds, cultured plant cells, protoplasts, calluses. Certain compounds like- DMSO (dimethyl sulfoxide), glycerol, ethylene, propylene, sucrose, mannose, glucose, praline, acetamide etc are added during the cryopreservation. These are called cryoprotectants and prevent the damage caused to cells (by freezing or thawing) by reducing the freezing point and super cooling point of water^[12].

(b) Cold Storage- Cold storage is a slow growth germplasm conservation method and conserves the germplasm at a low and non-freezing temperature (1-9°C). The growth of the plant material is slowed down in cold storage in contrast to complete stoppage in cryopreservation and thus prevents cryogenic injuries. Long term cold storage is simple, cost effective and yields germplasm with good survival rate. Virus free strawberry plants could be preserved at 10°C for about 6 years. Several grape plants have been stored for over 15 years by using a cold storage at temperature around 9°C and transferring them in the fresh medium every year.

(c) Low pressure and low oxygen storage- In low- pressure storage, the atmospheric pressure surrounding the plant material is reduced and in the low oxygen storage, the oxygen concentration is reduced. The lowered partial pressure reduces the in vitro growth of plants. In the low-oxygen storage, the oxygen concentration is reduced and the partial pressure of oxygen below 50 mmHg reduces plant tissue growth. Due to the reduced availability of O₂, and reduced production of CO₂, the photosynthetic activity is reduced which inhibits the plant tissue growth and dimension. This method has also helped in increasing the shelf life of many fruits, vegetables and flowers. The germplasm conservation through the conventional methods has several limitations such as short-lived seeds, seed dormancy, seed-borne diseases, and high inputs of cost and labour. The techniques of cryo-preservation (freezing cells and tissues at -196°C) and using cold storages help us to overcome these problems.

Production of Haploid Plants

Maheshwari and Guha ^[2] produce Haploid plants from anther culture of *Datura*. This marked the beginning of anther culture or pollen culture for the production of haploid plants. The technique has been further developed by Nitsch & Nitsch who isolated microspores of tobacco to produce complete plants. Application of haploidy has been used for the improvement of various plant species^[5].

CONCLUSION

Establishment of single cell cultures provides an excellent opportunity to investigate the properties and potentialities of plant cells. Such systems contribute to our understanding of the interrelationships and complementary influences of cells in multicellular organisms. These single cell systems have a great potential for crop improvement. Free cells in cultures permit quick administration and withdrawal of diverse chemicals/ substances, thereby making them easy targets for mutant selection. Moreover, the individual cells within a population of cultured cells invariably show cytogenetical and metabolic variations depending on the stage of the growth cycle and culture conditions. Organogenic differentiation is an outcome of the process of dedifferentiation followed by redifferentiation of cells. Dedifferentiation favors unorganized cell growth and the resultant developed callus has meristems randomly divided. Most of these meristems, if provided appropriate in vitro conditions, would redifferentiate shoot buds and roots (whole plant regeneration). This establishes the totipotency of somatic cells to undergo regeneration. Therefore, it is essential to understand the various types of cultures in vitro and also their growth patterns.

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