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Plant Tissue Culture: Current Status and Opportunities

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1. Introduction

Tissue culture is the *in vitro* aseptic culture of cells, tissues, organs or whole plant under controlled nutritional and environmental conditions [1] often to produce the clones of plants. The resultant clones are true-to type of the selected genotype. The controlled conditions provide the culture an environment conducive for their growth and multiplication. These conditions include proper supply of nutrients, pH medium, adequate temperature and proper gaseous and liquid environment.

Plant tissue culture technology is being widely used for large scale plant multiplication. Apart from their use as a tool of research, plant tissue culture techniques have in recent years, become of major industrial importance in the area of plant propagation, disease elimination, plant improvement and production of secondary metabolites. Small pieces of tissue (named explants) can be used to produce hundreds and thousands of plants in a continuous process. A single explant can be multiplied into several thousand plants in relatively short time period and space under controlled conditions, irrespective of the season and weather on a year round basis [2]. Endangered, threatened and rare species have successfully been grown and conserved by micropropagation because of high coefficient of multiplication and small demands on number of initial plants and space.

In addition, plant tissue culture is considered to be the most efficient technology for crop improvement by the production of somaclonal and gametoclonal variants. The micropropagation technology has a vast potential to produce plants of superior quality, isolation of useful variants in well-adapted high yielding genotypes with better disease resistance and stress tolerance capacities [3]. Certain type of callus cultures give rise to clones that have inheritable characteristics different from those of parent plants due to the possibility of occurrence of somaclonal variability [4], which leads to the development of commercially important improved varieties. Commercial production of

plants through micropropagation techniques has several advantages over the traditional methods of propagation through seed, cutting, grafting and air-layering etc. It is rapid propagation processes that can lead to the production of plants virus free [5]. *Corydalis yanhusuo*, an important medicinal plant was propagated by somatic embryogenesis from tuber-derived callus to produce disease free tubers [6]. Meristem tip culture of banana plants devoid from banana bunchy top virus (BBTV) and brome mosaic virus (BMV) were produced [7]. Higher yields have been obtained by culturing pathogen free germplasm *in vitro*. Increase in yield up to 150% of virus-free potatoes was obtained in controlled conditions [8]. The main objective of writing this chapter is to describe the tissue culture techniques, various developments, present and future trends and its application in various fields.

2. History of plant tissue culture

The science of plant tissue culture takes its roots from the discovery of cell followed by propounding of cell theory. In 1838, Schleiden and Schwann proposed that cell is the basic structural unit of all living organisms. They visualized that cell is capable of autonomy and therefore it should be possible for each cell if given an environment to regenerate into whole plant. Based on this premise, in 1902, a German physiologist, Gottlieb Haberlandt for the first time attempted to culture isolated single palisade cells from leaves in Knop's salt solution enriched with sucrose. The cells remained alive for up to one month, increased in size, accumulated starch but failed to divide. Though he was unsuccessful but laid down the foundation of tissue culture technology for which he is regarded as the father of plant tissue culture. After that some of the landmark discoveries took place in tissue culture which are summarized as under:

- 1902 - Haberlandt proposed concept of *in vitro* cell culture
- 1904 - Hannig cultured embryos from several cruciferous species
- 1922 - Kolte and Robbins successfully cultured root and stem tips respectively
- 1926 - Went discovered first plant growth hormone –Indole acetic acid
- 1934 - White introduced vitamin B as growth supplement in tissue culture media for tomato root tip
- 1939 - Gautheret, White and Nobecourt established endless proliferation of callus cultures
- 1941 - Overbeek was first to add coconut milk for cell division in *Datura*
- 1946 - Ball raised whole plants of *Lupinus* by shoot tip culture
- 1954 - Muir was first to break callus tissues into single cells
- 1955 - Skoog and Miller discovered kinetin as cell division hormone
- 1957 - Skoog and Miller gave concept of hormonal control (auxin: cytokinin) of organ formation
- 1959 - Reinert and Steward regenerated embryos from callus clumps and cell suspension of carrot (*Daucus carota*)
- 1960 - Cocking was first to isolate protoplast by enzymatic degradation of cell wall

- 1960 - Bergmann filtered cell suspension and isolated single cells by plating
- 1960 - Kanta and Maheshwari developed test tube fertilization technique
- 1962 - Murashige and Skoog developed MS medium with higher salt concentration
- 1964 - Guha and Maheshwari produced first haploid plants from pollen grains of *Datura* (Anther culture)
- 1966 - Steward demonstrated totipotency by regenerating carrot plants from single cells of tomato
- 1970 - Power *et al.* successfully achieved protoplast fusion
- 1971 - Takebe *et al.* regenerated first plants from protoplasts
- 1972 - Carlson produced first interspecific hybrid of *Nicotiana tabacum* by protoplast fusion
- 1974 - Reinhardt introduced biotransformation in plant tissue cultures
- 1977 - Chilton *et al.* successfully integrated Ti plasmid DNA from *Agrobacterium tumefaciens* in plants
- 1978- Melchers *et al.* carried out somatic hybridization of tomato and potato resulting in pomato
- 1981- Larkin and Scowcroft introduced the term somaclonal variation
- 1983 - Pelletier *et al.* conducted intergeneric cytoplasmic hybridization in Radish and Grape
- 1984 - Horsh *et al.* developed transgenic tobacco by transformation with *Agrobacterium*
- 1987 - Klien *et al.* developed biolistic gene transfer method for plant transformation
- 2005 - Rice genome sequenced under International Rice Genome Sequencing Project

3. Basics of plant cell and tissue culture

In plant cell culture, plant tissues and organs are grown *in vitro* on artificial media, under aseptic and controlled environment. The technique depends mainly on the concept of totipotentiality of plant cells [9] which refers to the ability of a single cell to express the full genome by cell division. Along with the totipotent potential of plant cell, the capacity of cells to alter their metabolism, growth and development is also equally important and crucial to regenerate the entire plant [1]. Plant tissue culture medium contains all the nutrients required for the normal growth and development of plants. It is mainly composed of macronutrients, micronutrients, vitamins, other organic components, plant growth regulators, carbon source and some gelling agents in case of solid medium [10]. Murashige and Skoog medium (MS medium) is most extensively used for the vegetative propagation of many plant species *in vitro*. The pH of the media is also important that affects both the growth of plants and activity of plant growth regulators. It is adjusted to the value between 5.4 - 5.8. Both the solid and liquid medium can be used for culturing. The composition of the medium, particularly the plant hormones and the nitrogen source has profound effects on the response of the initial explant.

Plant growth regulators (PGR's) play an essential role in determining the development pathway of plant cells and tissues in culture medium. The auxins, cytokinins and

gibberellins are most commonly used plant growth regulators. The type and the concentration of hormones used depend mainly on the species of the plant, the tissue or organ cultured and the objective of the experiment [11]. Auxins and cytokinins are most widely used plant growth regulators in plant tissue culture and their amount determined the type of culture established or regenerated. The high concentration of auxins generally favors root formation, whereas the high concentration of cytokinins promotes shoot regeneration. A balance of both auxin and cytokinin leads to the development of mass of undifferentiated cells known as callus.

Maximum root induction and proliferation was found in *Stevia rebaudiana*, when the medium is supplemented with 0.5 mg/l NAA [12]. Cytokinins generally promote cell division and induce shoot formation and axillary shoot proliferation. High cytokinin to auxin ratio promotes shoot proliferation while high auxin to cytokinins ratio results in root formation [13]. Shoot initiation and proliferation was found maximum, when the callus of black pepper was shifted to medium supplemented with BA at the concentration of 0.5 mg/l [14]. Gibberellins are used for enhanced growth and to promote cell elongation. Maximum shoot length was observed in *Phalaenopsis* orchids when cultured in medium containing 0.5 mg/l GA₃ (unpublished).

4. Tissue culture in agriculture

As an emerging technology, the plant tissue culture has a great impact on both agriculture and industry, through providing plants needed to meet the ever increasing world demand. It has made significant contributions to the advancement of agricultural sciences in recent times and today they constitute an indispensable tool in modern agriculture [5].

Biotechnology has been introduced into agricultural practice at a rate without precedent. Tissue culture allows the production and propagation of genetically homogeneous, disease-free plant material [37]. Cell and tissue *in vitro* culture is a useful tool for the induction of somaclonal variation [38]. Genetic variability induced by tissue culture could be used as a source of variability to obtain new stable genotypes. Interventions of biotechnological approaches for *in vitro* regeneration, mass micropropagation techniques and gene transfer studies in tree species have been encouraging. *In vitro* cultures of mature and/or immature zygotic embryos are applied to recover plants obtained from inter-generic crosses that do not produce fertile seeds [39]. Genetic engineering can make possible a number of improved crop varieties with high yield potential and resistance against pests. Genetic transformation technology relies on the technical aspects of plant tissue culture and molecular biology for:

- Production of improved crop varieties
- Production of disease-free plants (virus)
- Genetic transformation
- Production of secondary metabolites
- Production of varieties tolerant to salinity, drought and heat stresses

5. Germplasm conservation

In vitro cell and organ culture offers an alternative source for the conservation of endangered genotypes [40]. Germplasm conservation worldwide is increasingly becoming an essential activity due to the high rate of disappearance of plant species and the increased need for safeguarding the floristic patrimony of the countries [41]. Tissue culture protocols can be used for preservation of vegetative tissues when the targets for conservation are clones instead of seeds, to keep the genetic background of a crop and to avoid the loss of the conserved patrimony due to natural disasters, whether biotic or abiotic stress [42]. The plant species which do not produce seeds (sterile plants) or which have 'recalcitrant' seeds that cannot be stored for long period of time can successfully be preserved via *in vitro* techniques for the maintenance of gene banks.

Cryopreservation plays a vital role in the long-term *in vitro* conservation of essential biological material and genetic resources. It involves the storage of *in vitro* cells or tissues in liquid nitrogen that results in cryo-injury on the exposure of tissues to physical and chemical stresses. Successful cryopreservation is often ascertained by cell and tissue survival and the ability to re-grow or regenerate into complete plants or form new colonies [43]. It is desirable to assess the genetic integrity of recovered germplasm to determine whether it is 'true-to-type' following cryopreservation [44]. The fidelity of recovered plants can be assessed at phenotypic, histological, cytological, biochemical and molecular levels, although, there are advantages and limitations of the various approaches used to assess genetic stability [45]. Cryobionomics is a new approach to study genetic stability in the cryopreserved plant materials [46]. The embryonic tissues can be cryopreserved for future use or for germplasm conservation [47].

6. Embryo culture

Embryo culture is a type of plant tissue culture that is used to grow embryos from seeds and ovules in a nutrient medium. In embryo culture, the plant develops directly from the embryo or indirectly through the formation of callus and then subsequent formation of shoots and roots. The technique has been developed to break seed dormancy, test the vitality of seeds, production of rare species and haploid plants [59, 119]. It is an effective technique that is employed to shorten the breeding cycle of plants by growing excised embryos and results in the reduction of long dormancy period of seeds. Intra-varietal hybrids of an economically important energy plant "Jatropha" have been produced successfully with the specific objective of mass multiplication [62]. Somatic embryogenesis and plant regeneration has been carried out in embryo cultures of Jucara Palm for rapid cloning and improvement of selected individuals [60]. In addition, conservation of endangered species can also be attained by practicing embryo culture technique. Recently a successful protocol has been developed for the *in vitro* propagation of *Khaya grandifoliola* by excising embryos from mature seeds [61]. The plant has a high economic value for timber wood and for medicinal purposes as well. This technique has an important application in forestry by offering a mean of propagation of elite individuals where the selection and improvement of natural population is difficult.

7. Genetic transformation

Genetic transformation is the most recent aspect of plant cell and tissue culture that provides the mean of transfer of genes with desirable trait into host plants and recovery of transgenic plants [63]. The technique has a great potential of genetic improvement of various crop plants by integrating in plant biotechnology and breeding programmes. It has a promising role for the introduction of agronomically important traits such as increased yield, better quality and enhanced resistance to pests and diseases [64].

Genetic transformation in plants can be achieved by either vector-mediated (indirect gene transfer) or vector less (direct gene transfer) method [65]. Among vector dependant gene transfer methods, *Agrobacterium*-mediated genetic transformation is most widely used for the expression of foreign genes in plant cells. Successful introduction of agronomic traits in plants was achieved by using root explants for the genetic transformation [66]. Virus-based vectors offers an alternative way of stable and rapid transient protein expression in plant cells thus providing an efficient mean of recombinant protein production on large scale [67].

Recently successful transgenic plants of *Jatropha* were obtained by direct DNA delivery to mature seed-derived shoot apices via particle bombardment method [68]. This technology has an important impact on the reduction of toxic substances in seeds [69] thus overcoming the obstacle of seed utilization in various industrial sector. Regeneration of disease or viral resistant plants is now achieved by employing genetic transformation technique. Researchers succeeded in developing transgenic plants of potato resistant to potato virus Y (PVY) which is a major threat to potato crop worldwide [70]. In addition, marker free transgenic plants of *Petunia hybrida* were produced using multi-auto-transformation (MAT) vector system. The plants exhibited high level of resistance to *Botrytis cinerea*, causal agent of gray mold [71].

8. Protoplast fusion

Somatic hybridization is an important tool of plant breeding and crop improvement by the production of interspecific and intergeneric hybrids. The technique involves the fusion of protoplasts of two different genomes followed by the selection of desired somatic hybrid cells and regeneration of hybrid plants [48]. Protoplast fusion provides an efficient mean of gene transfer with desired trait from one species to another and has an increasing impact on crop improvement [3]. Somatic hybrids were produced by fusion of protoplasts from rice and ditch reed using electrofusion treatment for salt tolerance [49].

In vitro fusion of protoplast opens a way of developing unique hybrid plants by overcoming the barriers of sexual incompatibility. The technique has been applicable in horticultural industry to create new hybrids with increased fruit yield and better resistance to diseases. Successful viable hybrid plants were obtained when protoplasts from citrus were fused with other related citrinae species [50]. The potential of somatic hybridization in important crop plants is best illustrated by the production of intergeneric hybrid plants among the members of Brassicaceae [51]. To resolve the problem of loss of chromosomes and decreased

regeneration capacity, successful protocol has been established for the production of somatic hybrid plants by using two types of wheat protoplast as recipient and protoplast of *Haynaldia villosa* as a fusion donor. It is also employed as an important gene source for wheat improvement [52].

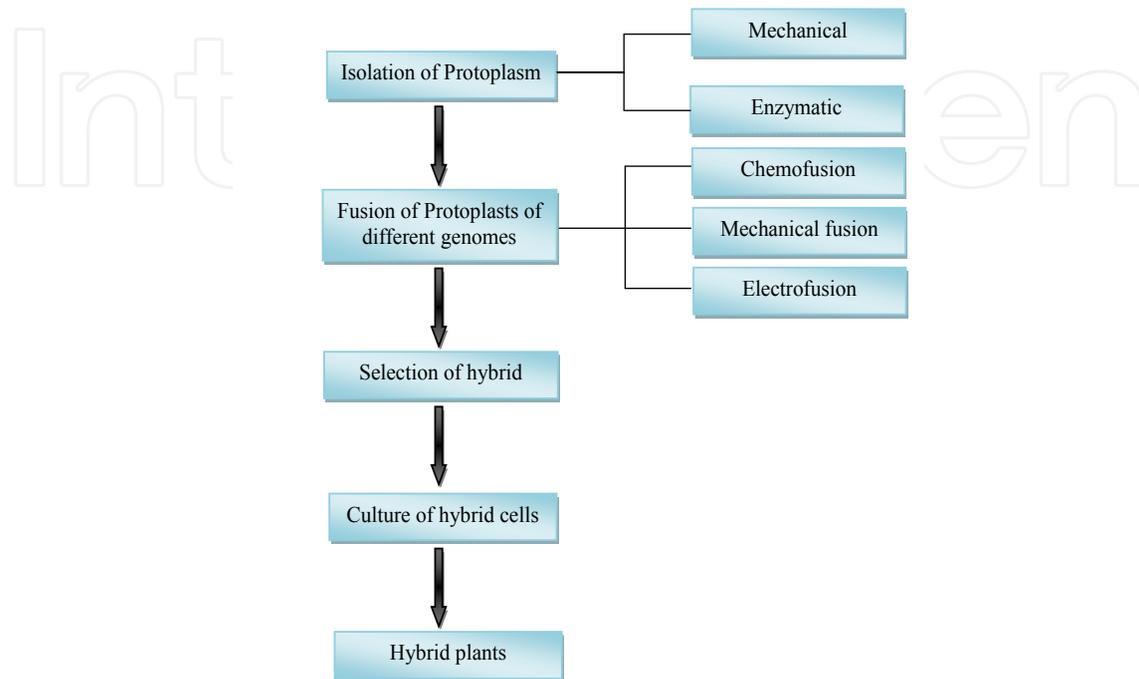


Figure 1. Schematic representation of production of hybrid plant via protoplast fusion

9. Haploid production

The tissue culture techniques enable to produce homozygous plants in relatively short time period through the protoplast, anther and microspore cultures instead of conventional breeding [53].

Haploids are sterile plants having single set of chromosomes which are converted into homozygous diploids by spontaneous or induced chromosome doubling. The doubling of chromosomes restores the fertility of plants resulting in production of double haploids with potential to become pure breeding new cultivars [54]. The term androgenesis refers to the production of haploid plants from young pollen cells without undergoing fertilization. Sudherson *et al.* [55] reported haploid plant production of sturt's desert pea by using pollen grains as primary explants. The haploidy technology has now become an integral part of plant breeding programs by speeding up the production of inbred lines [56] and overcoming the constraints of seed dormancy and embryo non-viability [57]. The technique has a remarkable use in genetic transformation by the production of haploid plants with induced resistance to various biotic and abiotic stresses. Introduction of genes with desired trait at haploid state followed by chromosome doubling led to the production of double haploids inbred wheat and drought tolerant plants were attained successfully [58].

10. Current and future status of plant tissue culture

The past decades of plant cell biotechnology has evolved as a new era in the field of biotechnology, focusing on the production of a large number of secondary plant products. During the second half of the last century the development of genetic engineering and molecular biology techniques allowed the appearance of improved and new agricultural products which have occupied an increasing demand in the productive systems of several countries worldwide [31, 32, 33, 34]. Nevertheless, these would have been impossible without the development of tissue culture techniques, which provided the tools for the introduction of genetic information into plant cells [35]. Nowadays, one of the most promising methods of producing proteins and other medicinal substances, such as antibodies and vaccines, is the use of transgenic plants [36]. Transgenic plants represent an economical alternative to fermentation-based production systems. Plant-made vaccines or antibodies (plantibodies) are especially striking, as plants are free of human diseases, thus reducing screening costs for viruses and bacterial toxins. The number of farmers who have incorporated transgenic plants into their production systems in 2008 was 13.3 million, in comparison to 11 million in 2007 [34].

11. Techniques of plant tissue culture

11.1. Micropropagation

Micropropagation starts with the selection of plant tissues (explant) from a healthy, vigorous mother plant [15]. Any part of the plant (leaf, apical meristem, bud and root) can be used as explant. The whole process can be summarized into the following stages as shown in Figure 2.

11.2. Stage 0: Preparation of donor plant

Any plant tissue can be introduced *in vitro*. To enhance the probability of success, the mother plant should be *ex vitro* cultivated under optimal conditions to minimize contamination in the *in vitro* culture [16].

11.3. Stage I: Initiation stage

In this stage an explant is surface sterilized and transferred into nutrient medium. Generally, the combined application of bactericide and fungicide products is suggested. The selection of products depends on the type of explant to be introduced. The surface sterilization of explant in chemical solutions is an important step to remove contaminants with minimal damage to plant cells [17]. The most commonly used disinfectants are sodium hypochlorite [18, 19], calcium hypochlorite [20], ethanol [21] and mercuric chloride (HgCl_2) [17]. The cultures are incubated in growth chamber either under light or dark conditions according to the method of propagation.

11.4. Stage II: Multiplication stage

The aim of this phase is to increase the number of propagules [22]. The number of propagules is multiplied by repeated subcultures until the desired (or planned) number of plants is attained.

11.5. Stage III: Rooting stage

The rooting stage may occur simultaneously in the same culture media used for multiplication of the explants. However, in some cases it is necessary to change media, including nutritional modification and growth regulator composition to induce rooting and the development of strong root growth.

11.6. Stage IV: Acclimatization Stage

At this stage, the *in vitro* plants are weaned and hardened. Hardening is done gradually from high to low humidity and from low light intensity to high light intensity. The plants are then transferred to an appropriate substrate (sand, peat, compost etc.) and gradually hardened under greenhouse.

12. Somatic embryogenesis and organogenesis

Somatic embryogenesis: is an *in vitro* method of plant regeneration widely used as an important biotechnological tool for sustained clonal propagation [23]. It is a process by which somatic cells or tissues develop into differentiated embryos. These somatic embryos can develop into whole plants without undergoing the process of sexual fertilization as done by zygotic embryos. The somatic embryogenesis can be initiated directly from the explants or indirectly by the establishment of mass of unorganized cells named callus.

Plant regeneration via somatic embryogenesis occurs by the induction of embryogenic cultures from zygotic seed, leaf or stem segment and further multiplication of embryos. Mature embryos are then cultured for germination and plantlet development, and finally transferred to soil

Somatic embryogenesis has been reported in many plants including trees and ornamental plants of different families. The phenomenon has been observed in some cactus species [24]. There are various factors that affect the induction and development of somatic embryos in cultured cells. A highly efficient protocol has been reported for somatic embryogenesis on grapevine [25] that showed higher plant regeneration sufficiently when the tissues were cultured in liquid medium. Plant growth regulators play an important role in the regeneration and proliferation of somatic embryos. Highest efficiency of embryonic callus was induced by culturing nodal stem segments of rose hybrids on medium supplemented with various PGR's alone or in combination [26]. This embryonic callus showed high germination rate of somatic embryos when grown on abscisic acid

(ABA) alone. Somatic embryogenesis is not only a process of regenerating the plants for mass propagation but also regarded as a valuable tool for genetic manipulation. The process can also be used to develop the plants that are resistant to various kinds of stresses [27] and to introduce the genes by genetic transformation [28]. A successful protocol has been developed for regeneration of cotton cultivars with resistance to *Fusarium* and *Verticillium* wilts [29].

Organogenesis: refers to the production of plant organs i.e. roots, shoots and leaves that may arise directly from the meristem or indirectly from the undifferentiated cell masses (callus). Plant regeneration via organogenesis involves the callus production and differentiation of adventitious meristems into organs by altering the concentration of plant growth hormones in nutrient medium. Skoog and Muller [30] were the first who demonstrated that high ratio of cytokinin to auxin stimulated the formation of shoots in tobacco callus while high auxin to cytokinin ratio induced root regeneration.

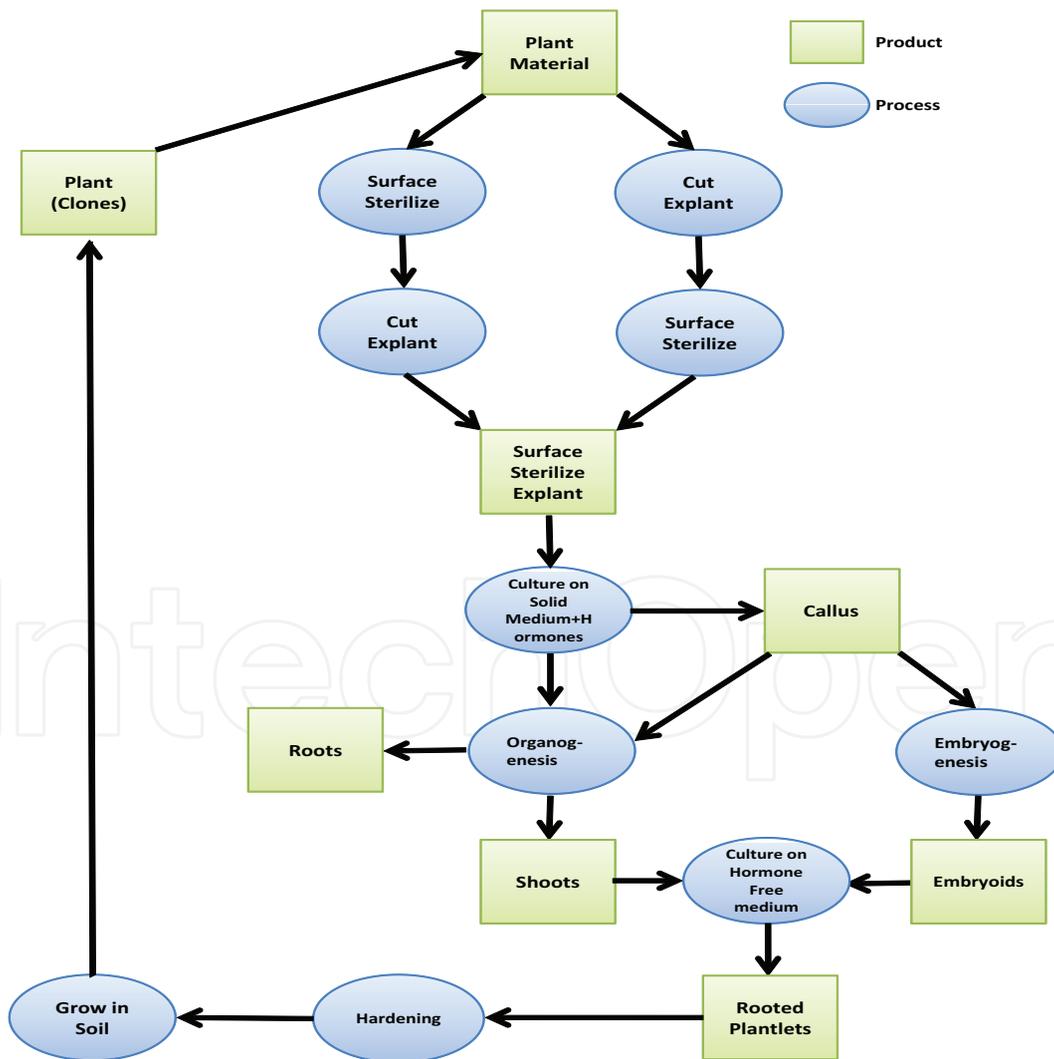


Figure 2. Flow chart summarizing tissue culture experiments.

13. Tissue culture in pharmaceuticals

Plant cell and tissue cultures hold great promise for controlled production of myriad of useful secondary metabolites [72]. Plant cell cultures combine the merits of whole-plant systems with those of microbial and animal cell cultures for the production of valuable therapeutic secondary metabolites [73]. In the search for alternatives to production of medicinal compounds from plants, biotechnological approaches, specifically plant tissue cultures, are found to have potential as a supplement to traditional agriculture in the industrial production of bioactive plant metabolites [74]. Exploration of the biosynthetic capabilities of various cell cultures has been carried out by a group of plant scientists and microbiologists in several countries during the last decade [75].

Cell suspension culture: Cell suspension culture systems are used now days for large scale culturing of plant cells from which secondary metabolites could be extracted. A suspension culture is developed by transferring the relatively friable portion of the callus into liquid medium and is maintained under suitable conditions of aeration, agitation, light, temperature and other physical parameters [76]. Cell cultures cannot only yield defined standard phytochemicals in large volumes but also eliminate the presence of interfering compounds that occur in the field-grown plants [77]. The advantage of this method is that it can ultimately provide a continuous, reliable source of natural products [78]. The major advantage of the cell cultures include synthesis of bioactive secondary metabolites, running in controlled environment, independently from climate and soil conditions [79]. A number of different types of bioreactors have been used for mass cultivation of plant cells. The first commercial application of large scale cultivation of plant cells was carried out in stirred tank reactors of 200 liter and 750 liter capacities to produce shikonin by cell culture of *Lithospermum erythrorhizon* [80]. Cell of *Catharanthus roseus*, *Dioscorea deltoidea*, *Digitalis lanata*, *Panax notoginseng*, *Taxus wallichiana* and *Podophyllum hexandrum* have been cultured in various bioreactors for the production of secondary plant products.

A number of medicinally important alkaloids, anticancer drugs, recombinant proteins and food additives are produced in various cultures of plant cell and tissues. Advances in the area of cell cultures for the production of medicinal compounds has made possible the production of a wide variety of pharmaceuticals like alkaloids, terpenoids, steroids, saponins, phenolics, flavanoids and amino acids [72, 81]. Some of these are now available commercially in the market for example shikonin and paclitaxel (Taxol). Until now 20 different recombinant proteins have been produced in plant cell culture, including antibodies, enzymes, edible vaccines, growth factors and cytokines [73]. Advances in scale-up approaches and immobilization techniques contribute to a considerable increase in the number of applications of plant cell cultures for the production of compounds with a high added value. Some of the secondary plant products obtained from cell suspension culture of various plants are given in Table 1.

Secondary metabolite	Plant name	Reference
Vasine	<i>Adhatoda vasica</i>	[82]
Artemisinin	<i>Artemisia annua</i>	[83]
Azadirachtin	<i>Azadirachta indica</i>	[84]
Cathin	<i>Brucea javanica</i>	[85]
Capsiacin	<i>Capsicum annum</i>	[86]
Senosides	<i>Cassia senna</i>	[87]
Ajmalicine	<i>Catharanthus roseus</i>	[88]
Secologanin		[89]
Indole alkaloids		[90]
Vincristine		[91]
Stilbenes	<i>Cayratia trifoliata</i>	[92]
Berberin	<i>Cosciniun fenustratum</i>	[93]
Sterols	<i>Hyssopus officinalis</i>	[94]
Shikonin	<i>Lithospermum erythrorhizon</i>	[95]
Ginseng saponin	<i>Panax notoginseng</i>	[96]
Podophyllotoxin	<i>Podophyllum hexandrum</i>	[97]
Taxane Paclitaxel	<i>Taxus chinensis</i>	[98]

Table 1. List of some secondary plant product produced in suspension culture

14. Hairy root cultures

The hairy root system based on inoculation with *Agrobacterium rhizogenes* has become popular in the last two decades as a method of producing secondary metabolites synthesized in plant roots [99]. Organized cultures, and especially root cultures, can make a significant contribution in the production of secondary metabolites. Most of the research efforts that use differentiated cultures instead of cell suspension cultures have focused on transformed (hairy) roots. *Agrobacterium rhizogenes* causes hairy root disease in plants. The neoplastic (cancerous) roots produced by *A. rhizogenes* infection are characterized by high growth rate, genetic stability and growth in hormone free media [100]. High stability [101] and productivity features allow the exploitation of hairy roots as valuable biotechnological tool for the production of plant secondary metabolites [102]. These genetically transformed root cultures can produce levels of secondary metabolites comparable to that of intact plants [103]. Hairy root technology has been strongly improved by increased knowledge of molecular mechanisms underlying their development. Optimizing the composition of nutrients for hairy root cultures is critical to gain a high production of secondary metabolites [100]. Some of the secondary plant products obtained from hairy root culture of various plants are shown in Table 2.

Secondary metabolite	Plant name	Reference
Rosmarinic acid	<i>Agastache rugosa</i>	[104]
Deoursin	<i>Angelica gigas</i>	[105]
Resveratol	<i>Arachys hypogaea</i>	[106]
Tropane	<i>Brugmansia candida</i>	[107]
Asiaticoside	<i>Centella asiatica</i>	[108]
Rutin	<i>Fagopyrum esculentum</i>	[104]
Glucoside	<i>Gentiana macrophylla</i>	[109]
Glycyrrhizin	<i>Glycyrrhiza glabra</i>	[110]
Shikonin	<i>Lithospermum erythrorhizon</i>	[111]
Glycoside	<i>Panax ginseng</i>	[112]
Plumbagin	<i>Plumbago zeylanica</i>	[113]
Anthraquinone	<i>Rubia akane</i>	[114]
Silymarin	<i>Silybium marianum</i>	[115]
Flavonolignan	<i>Silybium mariyanm</i>	[116]
Vincamine	<i>Vinca major</i>	[117]
Withanoloid A	<i>Withania somnifera</i>	[118]

Table 2. List of some secondary plant product produced in Hairy root culture

15. Tissue culture facilities at Qarshi industries

Plant tissue culture Lab was established in 2004 with the objectives to raise endangered medicinal plant species and the plants difficult to raise through traditional methods for conservation and mass propagation. We have so far propagated 12 medicinal plant species (*Plumbago zeylanica* L., *Nicotiana tabacum* L., *Artemisia absinthium* L., *Rosa damascena* Mill., *Althea rosea* L., *Stevia rebaudiana* Bertoni., *Jatropha curcas* L., *Phalaenopsis*, *Piper nigrum* L., *Solanum tuberosum* L., *Araucaria heterophylla* Salisb. Franco., *Taxus wallichiana* Zucc.) and currently working on propagation of commercially important endangered woody plant species like *Taxus wallichiana*. Commercialization of some fruit and vegetable crops are underway. The protocols developed for The Moth Orchid, Tobacco, Honey Plant, Potato and Physic nut are presented as case studies.

16. Case study 1

16.1. Micropropagation of *Phalaenopsis* "The Moth Orchids"

Orchids are usually grown for the beauty, exoticism and fragrance of their flowers. They are cultivated since the times of Confucius (ca. 551 - 479 BC). Some orchids are commercialized not for their beauty, but for uses in food industry. They are also used medicinally as a treatment for diarrhea and as an aphrodisiac. The vegetative propagation of *phalaenopsis* is difficult and time consuming. In addition, the desired characteristics of seedlings and uniformity are not attained.

In vitro propagation studies of phalaenopsis “the moth orchids” had the objective to develop a protocol for plant regeneration from callus. Thus *in vitro* culture techniques are adopted for quick propagation of commercially important orchid species. Regeneration from callus gives a way to rectify the problem of explants shortage. The callus of phalaenopsis previously obtained from the mature orchid plant was used as explant source. The callus was maintained on MS medium added with 3.0 % sucrose, 0.8 % agar, and different concentrations of BAP and 2, 4-D. Callus was sub-cultured after every 30 days for proliferation. Maximum callus proliferation was obtained when the medium was supplemented with 0.5 mg/l BA. Fresh green and non friable callus was obtained. For shoot regeneration and elongation, the callus was transferred to MS medium supplemented with BAP and GA₃ at different concentrations. Maximum shoot elongation was obtained in medium supplemented with 1.0 mg/l GA₃ as shown in Figure 3 a, b, c.

The regenerated shoots showed excess root development when transferred to medium added with 2.0 mg/l IBA. Further research work will focus on different potting medium compositions best suited for acclimatization of regenerated plants. As a high value crop, the mass production of orchids will provide a good opportunity of marketing locally as a good source of income.

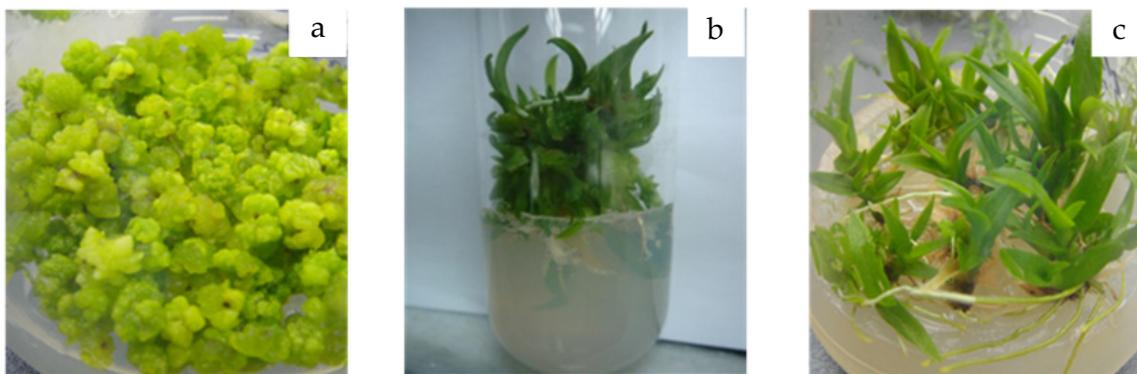


Figure 3. Micropropagation of Orchids (a) callus culture (b) shoot regeneration (c) rooted plantlets

17. Case study 2

17.1. Tissue culture of Tobacco (*Nicotiana tabacum* L.)

Tobacco is an important crop of Pakistan which covers a large area under cultivation. Being a cash crop grown all over the world, it has a good economic value. Fresh leaves of the plants are processed to obtain an agricultural product that is commercially available in dried, cured and natural forms. Clonal propagation of four important low nicotine content hybrid varieties of tobacco i.e. PGH-01, PGH-02, PGH-04 and PGH-09 was carried out with the special objective of commercialization of tissue cultured plants to the farmers and industry. The mother plants were provided by Pakistan Tobacco Board (PTB). Leaves and meristems were used as explants for the initiation of callus culture. Callus induction and proliferation was carried out on MS medium supplemented with different concentrations of

2,4-D. Excellent growth of callus was obtained at medium containing 1.0 mg/l 2,4-D. Callus was transferred to next medium for shoot regeneration. Efficient numbers of shoots were obtained when culture was shifted to MS medium supplemented with 0.5 mg/l BAP. For root induction different concentrations of IBA and NAA were tested and the result was found best on the same medium supplemented with 2.0 mg/l IBA as shown in Figure 4 a, b, c.

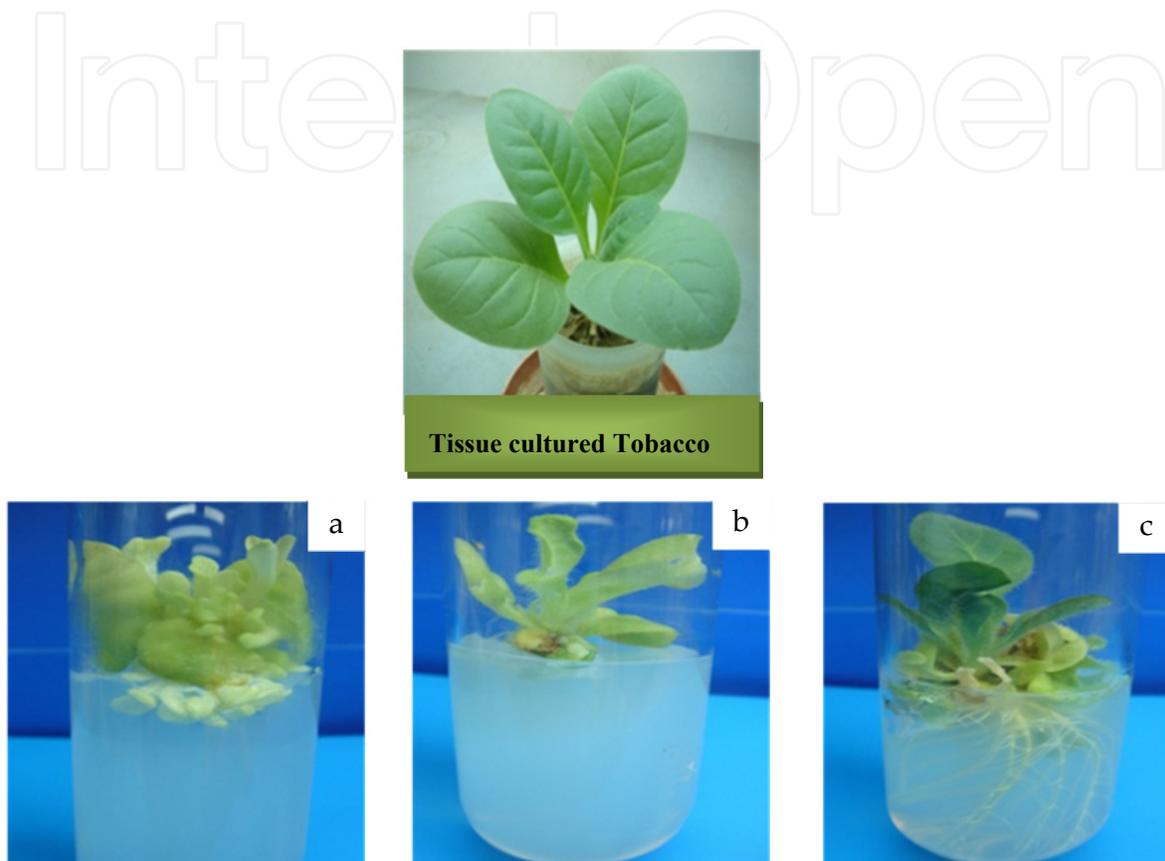


Figure 4. Tissue culture of *Nicotiana tabacum* (a) callus (b) shoot regeneration (c) root induction

18. Case study 3

***In vitro* propagation of Honey Plant (*Stevia rebaudiana* Bertoni)** The *in vitro* clonal propagation of *Stevia rebaudiana* was conducted by inoculating seeds on MS medium [10] and placing under photoperiod of 16 hrs light and 8 hrs dark in growth room. The seedlings with four nodes have been divided into 0.5 cm pieces of nodal segments and used as explants. For shoot multiplication, the nodal explants were inoculated on MS medium supplemented with 3.0% sucrose and 0.5, 1.0, 2.0, 3.0 and 4.0 mg/l of BAP and Kn (Kinetin) alone or in combinations with 0.25 and 0.5 mg/l of IAA. MS medium containing 2.0 mg/l BAP showed the best response to multiple shoot formation, while the highest shoot length (3.73 ± 0.14 cm) per micro shoot was observed on MS medium containing 2.0 Kn and 0.25 mg/l IAA after 15 days of inoculation as shown in Figure 5 a, b, c. Excised micro shoots were cultured on MS medium supplemented with 0.25, 0.5, 1.0 and 1.5 mg/l of NAA and IBA separately for the root induction. The optimal rooting (81%) was observed on MS medium

containing 0.5 mg/l NAA with 2 % sucrose within two weeks of culture transfer. The rooted plantlets were acclimatized successfully and transferred to greenhouse under low light intensity. This protocol for *in vitro* clonal propagation of *Stevia rebaudiana* has been optimized for the local environment, as a consequence it will be helpful to establish and cultivate *Stevia rebaudiana* for commercial scale production in various environmental conditions in Pakistan.



Figure 5. *In vitro* propagation of *S. rebaudiana* (a) seed germination on MS medium (b) shoot multiplication (c) root development.

19. Case study 4

19.1. Multiplication and regeneration of Potato (*Solanum tuberosum* L.) from nodal explants

Solanum tuberosum L. (potato) is the most important vegetable crop that occupies major area under cultivation in Pakistan. The crop is high yielding, has high nutritive value and gives maximum returns to farmers. Tissue culture is employed as a technique for rapid multiplication of potato plants free from diseases. The research was carried out with the objective of mass multiplication of true-to type three potato varieties i.e. Desiree, Diamant and Cardinal. The plant material for this research was provided by Four Brothers Agri Services Pakistan. The Company is working for introduction of high yielding vegetable & crop varieties in Pakistan.

The disease free potato tubers were washed both with detergent and distilled water to remove impurities and allowed to sprouting. Five days old sprouts were used as explants for direct proliferation. The explants were surface sterilized in detergent for 10 minutes, later with 0.1 % mercuric chloride solution for 5 minutes followed by three times washing with sterilized-distilled water. The sprouts were aseptically cut into 10 mm sections containing one node and inoculated in medium. The Espinosa medium plus vitamin B5 supplemented with different concentrations of BAP and GA3 alone and in combinations was utilized. Highest shoot length of shoots was observed in presence of 0.5 mg/l BAP and 0.4 mg/l GA3 with the ability to produce maximum plantlets per explant. For root induction the same medium was used with different concentrations of NAA and IBA. NAA at 2.0 mg/l induced the highest root development. The rooted plantlets were successfully acclimatized and delivered to the company for cultivation.

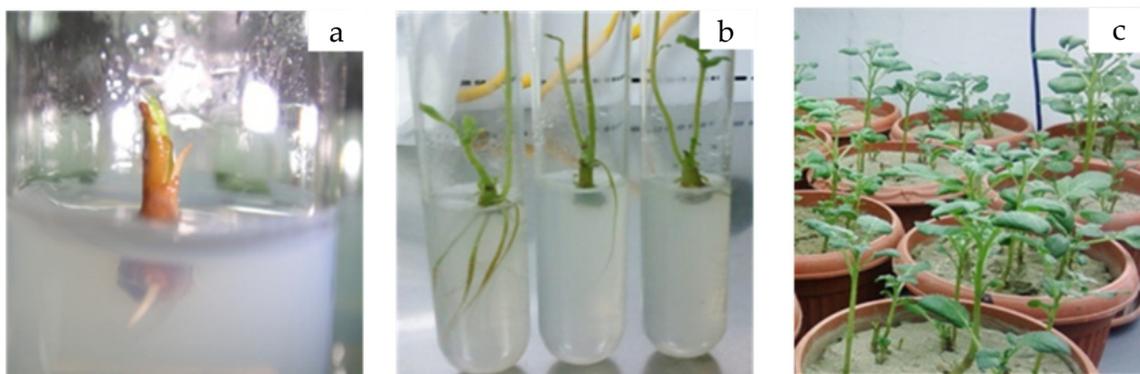


Figure 6. Tissue culture of Potato (a) nodal segment (b) regenerated shoots and roots (c) tissue cultured potato

20. Case study 5

20.1. Tissue culture of physic nut (*Jatropha curcas* L.)

The research studies on Tissue Culture of *Jatropha* (physic nut) had the objectives to develop protocol for mass propagation of elite trees selected on the bases of higher seed production and oil content. The experimental plant of *Jatropha curcas* was grown in the laboratory under controlled conditions for *in vitro* studies.

Leaf and apical meristem explants isolated from 7 days old seedling of *Jatropha curcas*, were use to induce callus.

Murashige & Skoog (1962) medium supplemented with different growth regulator formulations including 2,4-D and IBA was used. Excellent growth of callus on leaf explants was obtained in medium supplemented with 1.0 mg/L 2, 4-D. Callus produced from leaf explants in all IBA concentrations grew faster during 7 to 30 days of culture and then stabilized at a slow growth rate. While 1.0 mg/L 2,4-D was proved to be most effective in inducing callus on a large scale in short period of time. Callus was soft, friable and white in color.

Apical meristem was used as explant for direct shoot regeneration. Rooting from meristem was effectively achieved on MS supplemented with 1.5, 2.0 and 2.5 mg/l IBA. Root induction with 2.0 mg/l IBA was most effective and the roots also developed secondary roots.

In near future somatic embryogenesis and shoot regeneration from callus will be tested in MS medium supplemented with various concentrations of BA. The regenerated plant will be acclimatized and released for field planting under various climatic and soil conditions for further studies.

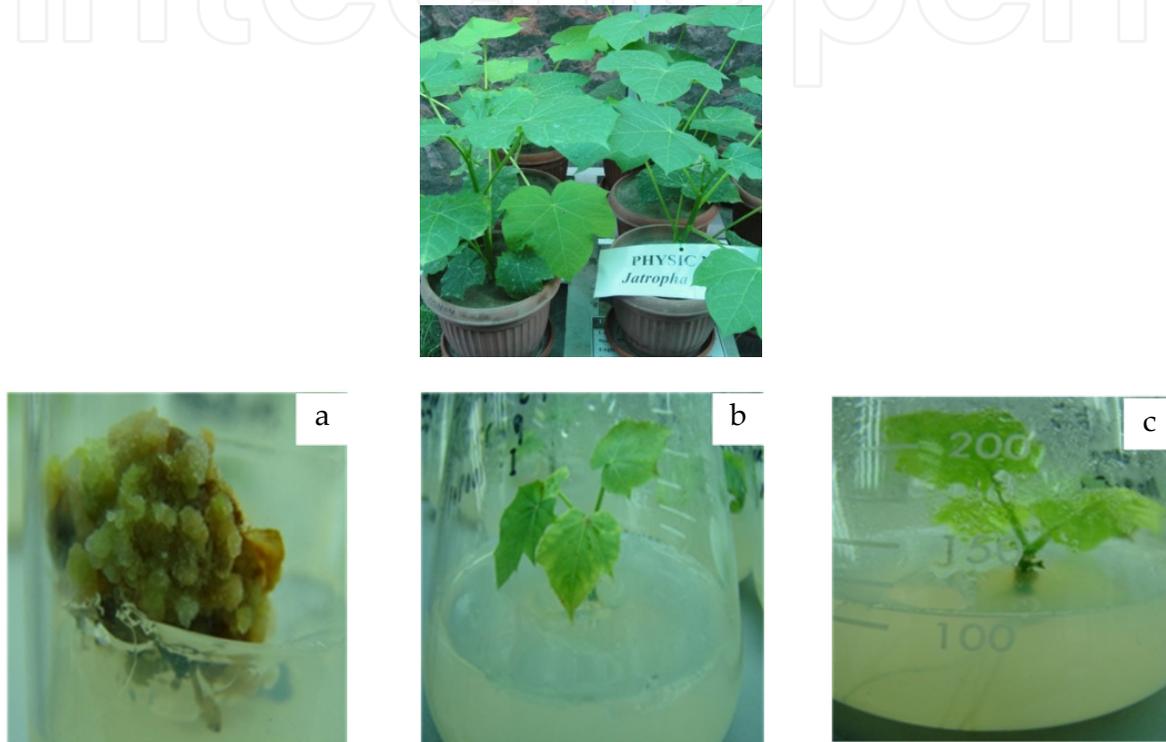


Figure 7. Tissue culture of *Jatropha curcas* (a) callus of *Jatropha* (b) shoot regeneration (c) root induction.

21. Conclusion

Plant tissue culture represents the most promising areas of application at present time and giving an out look into the future. The areas ranges from micropropagation of ornamental and forest trees, production of pharmaceutically interesting compounds, and plant breeding for improved nutritional value of staple crop plants, including trees to cryopreservation of valuable germplasm. All biotechnological approaches like genetic engineering, haploid induction, or somaclonal variation to improve traits strongly depend on an efficient *in-vitro* plant regeneration system.

The rapid production of high quality, disease free and uniform planting stock is only possible through micropropagation. New opportunities has been created for producers, farmers and nursery owners for high quality planting materials of fruits, ornamentals, forest tree species and vegetables. Plant production can be carried out throughout the year

irrespective of season and weather. However micropropagation technology is expensive as compared to conventional methods of propagation by means of seed, cuttings and grafting etc. Therefore it is essential to adopt measures to reduce cost of production. Low cost production of plants requires cost effective practices and optimal use of equipment to reduce the unit cost of plant production. It can be achieved by improving the process efficiency and better utilization of resources. Bioreactor based plant propagation can increase the speed of multiplication and growth of cultures and reduce space, energy and labor requirements when commencing commercial propagation. However, the use of bioreactors needs special care and handling to avoid contamination of culture which may lead to heavy economic losses. The cost of production may also be reduced by selecting several plants that provide the option for around the year production and allow cost flow and optimal use of equipment and resources. It is also essential to have sufficient mother culture and reduce the number of subculture to avoid variation and plan the production of plants according to the demand.

Quality control is also very essential to assure high quality plant production and to obtain confidence of the consumers. The selection of explants source, diseases free material, authenticity of variety and elimination of somaclonal variants are some of the most critical parameters for ensuring the quality of the plants.

The *in vitro* culture has a unique role in sustainable and competitive agriculture and forestry and has been successfully applied in plant breeding for rapid introduction of improved plants. Plant tissue culture has become an integral part of plant breeding. It can also be used for the production of plants as a source of edible vaccines. There are many useful plant-derived substances which can be produced in tissue cultures.

Since last two decades there have been considerable efforts made in the use of plant cell cultures in bioproduction, bioconversion or biotransformation and biosynthetic studies. The potential commercial production of pharmaceuticals by cell culture techniques depends upon detailed investigations into the biosynthetic sequence. There is great potential of cell culture to be use in the production of valuable secondary products. Plant tissue culture is a noble approach to obtain these substances in large scale.

Plant cell culture has made great advances. Perhaps the most significant role that plant cell culture has to play in the future will be in its association with transgenic plants. The ability to accelerate the conventional multiplication rate can be of great benefit to many countries where a disease or some climatic disaster wipes out crops. The loss of genetic resources is a common story when germplasm is held in field genebanks. Slow growth *in vitro* storage and cryopreservation are being proposed as solutions to the problems inherent in field genebanks. If possible, they can be used with field genebanks, thus providing a secure duplicate collection. They are the means by which future generations will be able to have access to genetic resources for simple conventional breeding programmes, or for the more complex genetic transformation work. As such, it has a great role to play in agricultural development and productivity.

13. Commonly used terms in tissue culture

Adventitious: development of organs such as buds, leaves, roots, shoots and somatic embryos from shoot and root tissues and callus.

Agar: Natural gelling agent made from algae

Aseptic technique: procedures used to prevent the introduction of microorganisms such as fungi, bacteria, viruses and phytoplasmas into cell, tissue and organ cultures, and cross contamination of cultures.

Autoclave: A machine capable of sterilizing by steam under pressure

Axenic culture: a culture without foreign or undesired life forms but may include the deliberate co-culture with different types of cells, tissues or organisms.

Callus: an unorganized mass of differentiated plant cells.

Cell culture: culture of cells or their maintenance *in vitro* including the culture of single cells.

Chemically defined medium: a nutritive solution or substrate for culturing cells in which each component is specified.

Clonal propagation: asexual multiplication of plants from a single individual or explant.

Clones: a group of plants propagated from vegetative parts, which have been derived by repeated propagation from a single individual. Clones are considered to be genetically uniform.

Contamination: infected by unwanted microorganisms in controlled environment

Cryopreservation: ultra-low temperature storage of cells, tissues, embryos and seeds.

Culture: A plant growing *in vitro* in a sterile environment

Differentiated: cultured cells that maintain all or much of the specialized structure and function typical of the cell type *in vivo*.

Embryo culture: *In vitro* culture of isolated mature or immature embryos.

Explant: an excised piece or part of a plant used to initiate a tissue culture.

Ex vitro: Organisms removed from tissue culture and transplanted; generally plants to soil or potting mixture.

Hormone: Generally naturally occurring chemicals that strongly affect plant growth

In Vitro: To be grown in glass

In Vivo: To be grown naturally

Laminar Flow Hood: An enclosed work area where the air is cleaned using HEPA filters

Medium: a solid or liquid nutritive solution used for culturing cells

Meristem: a group of undifferentiated cells situated at the tips of shoots, buds and roots, which divide actively and give rise to tissue and organs.

Micropropagation: multiplication of plants from vegetative parts by using tissue culture nutrient medium.

Propagule: a portion of an organism (shoot, leaf, callus, etc.) used for propagation.

Somatic embryos: non-zygotic bipolar embryo-like structures obtained from somatic cells.

Subculture: the aseptic division and transfer of a culture or portion of that culture to a fresh synthetic media.

Tissue culture: *in vitro* culture of cells, tissues, organs and plants under aseptic conditions on synthetic media.

Totipotency: capacity of plant cells to regenerate whole plants when cultured on appropriate media.

Transgenic: plants that have a piece of foreign DNA

Undifferentiated: cells that have not transformed into specialized tissues

14. Abbreviations

BAP	6-benylaminopurine
2,4-D	2,4-dichlorophenoxyacetic acid
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
GA₃	Gibberellic acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
NAA	Naphthaleneacetic acid
KN	Kinetin

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