1. TECHNIQUES FOR SELECTING CELL LINES

The selection of cell line becomes important. It includes the establishment of high-producing and fast-growing in-vitro cultures. It is possible to identify cell lines that can produce amounts of compounds equal or even higher than in the plant from which they derive. Moreover, increase of metabolite levels using mutants is possible, and selection of suitable analogues for this purpose could be an important factor in order to produce a variety of products.

Maximization of the production and accumulation of secondary metabolites by plant cultured cells requires production of new genotypes through protoplast fusion or genetic engineering; however, this presupposes the identification of the genes encoding key enzymes of secondary metabolic pathways and their expression. Use of mutagens increases the variability which already exists in living cells. Furthermore, new molecules, which have previously not been found in plants, can be produced by cell cultures.

IMMOBILIZATION OF PLANT CELLS

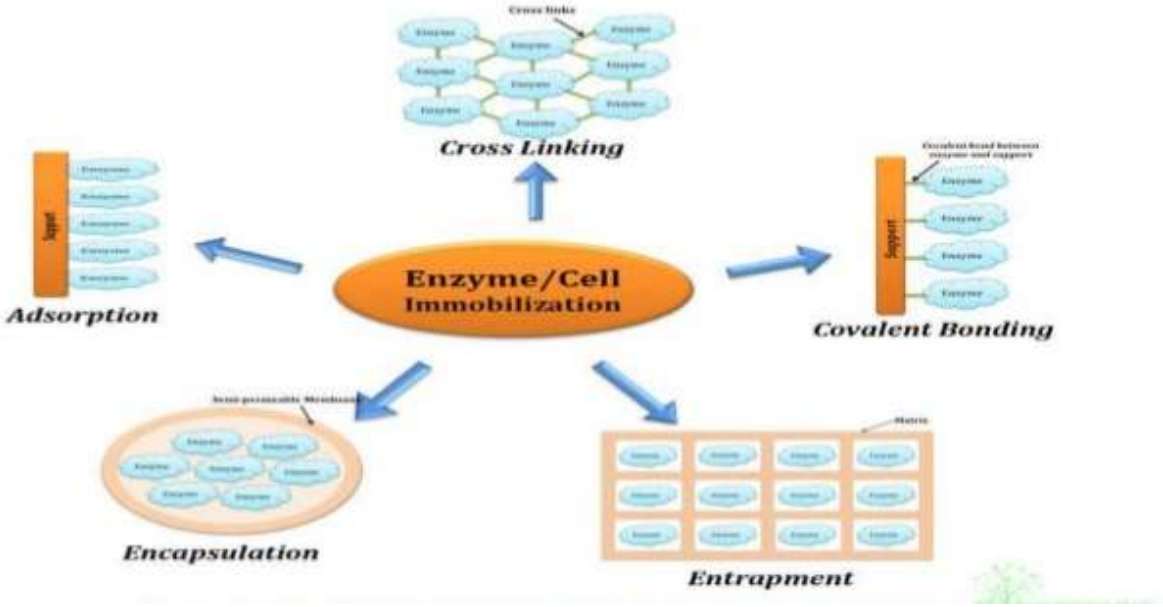
Decreasing cell mobility is an important factor in cell cultures. This can be achieved by entrapment of cell in alginate beads, polyurethane foams or by using gelling agents like agarose, agar, polyacrylamide or chitoson-carrageenan. Cell immobility has certain advantages such as cells are protected against morphological changes and any organization due to shear forces and contact with other cells respectively. The cells can also be reused. There are various examples where cell immobilization has positively affected the secondary metabolite production as well as their release into the culture medium. Under these conditions the cells can be reused. Cell immobilization is also important for biotransformation reactions for carrying out reactions in bioreactors.

Immobilization of plant cells is considered to be of importance in research and development in plant cell cultures, because of the potential benefits that could be provided:

- The extended viability of cells in the stationary (and producing) stage, enabling maintenance of biomass over a prolonged time period;
- Simplified downstream processing (if products are secreted);
- Promotion of differentiation, linked with enhanced secondary metabolism;
- Reducing the risk of contamination;
- reduced shear sensitivity (especially with entrapped cells);

• promotion of secondary metabolite secretion, in some cases;

• minimization of fluid viscosity increase,



PRECURSOR FEEDING

Precursor feeding has been an obvious and popular approach to increase secondary metabolites production in plant cell cultures. Precursor feeding: exogenous supply of a biosynthetic precursor to culture medium may also increase the yield of the desired product. This approach is useful when the precursors are inexpensive. With the basis of the knowledge on biosynthetic pathways, several organic compounds have been added to the culture medium in order to enhancing the synthesis of secondary metabolites. Precursor feeding is based on the idea that any compound, which is an intermediate, in or at the beginning of a secondary metabolite biosynthetic route, stands a good chance of increasing the yield of the final product

Some examples for precursor feeding amino acids have been added to cell suspension culture media for production of tropane alkaloids, indole alkaloids etc. Addition of phenylalanine to *Salvia officinalis* cell suspension cultures stimulated the production of rosmarinic acid (Ellis and Towers 1970) Feeding ferulic acid to cultures of *Vanilla planifolia* resulted in an increase in vanillin accumulation (Romagnoli and Knorr 1998) Rosmarinic acid

BIOTRANSFORMATION

The term biotransformation is generally applied to describe the conversion of a molecule, may be natural or synthetic, by using living cultures, by entrapping enzymes or cells, or by permeabilization of cells, into a new product. Plant cells growing in culture conditions have the potential to carry out some special reactions depending upon the nature of compounds added in the culture medium. This potential is generally exploited to transform normal inactive molecules into some valuable, therapeutically important compounds. The cells are capable of producing new compounds through some general reactions including reduction, oxidations, hydrolysis, hydroxylation, epoxidation and isomerization (Suga and Hirata, 1990).

To carry out a successful biotransformation it is required that the enzymes involved in the process should be present in the culture, the rate of formation of the product should be higher as compared to the rate of its metabolism, the substrate should be able to enter into the cell, both the precursor and the product formed should not be toxic to the culture, the product synthesized should be able to be released into the medium. Another strategy is to add a precursor that may be a primary or intermediate metabolite used in the biosynthetic route that is usually in limited amounts in natural cells.

ELICITATION

In vitro culture techniques for regeneration of plants are a cost-effective and much efficient alternative approach for production of secondary metabolites as compared to classical methods commonly used for this purpose. Besides other advantages, these techniques are the only sustainable and eco-friendly system that can be used for the production of complex phytochemicals naturally produced in plant species which are rare or are at the verge of extinction due to extensive use and are not easily cultivated in natural environmental conditions. The production of secondary metabolites through biotechnology needs a system with optimum growth conditions which can be effectively produced by elicitation. An elicitor is a compound added in plant cell cultures/a living system in small quantities to stimulate the production of a target compound. These elicitors are grouped in two categories, biotic and abiotic, on the basis of their chemical structure and their origin inside or outside the cells

The plant secondary metabolites are known to have different functions in plants. Some of these functions are important for growth and development of plants for example the anthocyanin pigments give colors to flowers and help to attract insects for pollination. Similarly the alkaloids produced in several plant species and glucosinolates produced in the

plants of family Brassicaceae are toxic or produce toxins in response to pathogen attack to protect the plant from microorganisms and herbivores. The *in vivo* biosynthesis of these compounds is strictly under genetic control and is often induced after pathogen attack. The primary signals that are recognized by the plant after any pathogenic attack are called elicitors, microbe-associated molecular patterns (MAMPs) or pathogen-associated molecular patterns (PAMPs). The compounds which act as elicitors may be derived from the bacterial cell wall or flagella, or from the damaged cell wall of the plant under attack or the signaling molecules from the oral secretions of insects.

All these elicitors trigger the signal transduction pathways in plants that in turn produce compounds that act as secondary signals. The three most important secondary signaling molecules are jasmonates, salicylic acid and ethylene. Synthesis of these compounds produces a network of signal transduction that in turn initiates a series of reactions that help the plant cells in physiologically adapting to the external stress. The signal transduction pathways involving jasmonates, ethylene and salicylic acid either act synergistically or antagonistically producing various responses, leading to complex defense response.

The most important of these signal molecules for inducing secondary metabolite synthesis are the jasmonates. These compounds have been found to enhance the biosynthesis of several secondary metabolites including phenylpropanoids, terpenoids, alkaloids, and glucosinolates in many plant species. Jasmonates have been extensively used to enhance the production of alkaloids in tobacco and *C. roseus*. The mechanism of action of jasmonates in signal transduction has been extensively studied using *Arabidopsis thaliana* as a model plant.

Elicitation is one of the best methods at present utilized for enhancing the biotechnological generation of bioactive compounds. Elicitors are molecules that enhance any sort of plant resistance, stimulating the reactions of secondary metabolism in plants necessary for the protection of cells and whole plant. As per their origin, elicitors can be separated into two groups: biotic and abiotic. Abiotic elicitors are mainly the substances of inorganic in nature and mainly include inorganic molecules, for example, salts or physical parameters. Inorganic chemicals like metal ions or salts have been utilized to enhance the synthesis of bioactive plant constituents by their ability to alter the secondary metabolism in plants. Various salts specifically $AlCl_3$, $AgNO_3$, $CdCl_2$, $CaCl_2$, $CuCl_2$, $CoCl_2$, KCl, $HgCl_2$, $NiSO_4$, $MgSO_4$, $VOSO_4$ and Zn particles can stimulate the production of secondary metabolites in culture systems for a large number of plant species, for example, adventitious roots, cell suspensions and hairy roots.

Several biotic elicitors are perceived by particular receptors attached to the cell membrane. This perception is then conveyed to the cell through a signal transduction system, causing changes that in turn prompt the production of phytoalexins. The reaction of the plant is controlled by many components, basically its hereditary qualities and physiological form. As a general rule, plant protection from any disease is controlled by pathogen avirulence (Avr) genes and resistance (R) genes of the plant. Though in cultivars with similar R genes the specific Avr products can trigger the defense responses, the general elicitors can activate defenses in more than one plant species.

As per the new idea of plant internal resistance system, specific defense reactions are activated when plant cells perceive the MAMPs, another term used for exogenous and general elicitors. On the other hand, a pathogen attack can activate the production of endogenous plant elicitors, named as danger-associated molecular patterns. The next level of sensitivity includes the identification of pathogen-produced effectors, previously known as specific elicitors, which have been identified in various families, including proteins, lipids and glycans.

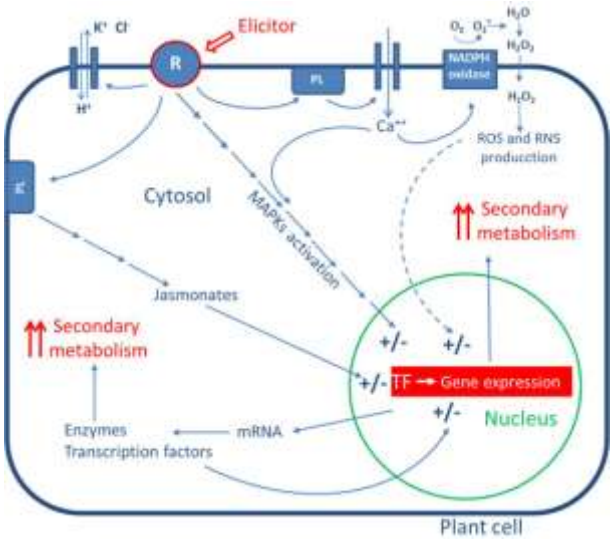


Figure Schematic representation of possible responses of cells to elicitation.

MAPKs: mitogen activated protein kinases; PL: phospholipase; R: receptor; ROS: reactive oxygen species; TF: transcription factors RNS: reactive nitrogen species.

BIOTIC ELICITORS

Originally the term elicitor was used to describe any molecule that can induce the synthesis of various compounds under different conditions in response to or under the stimulus of

various types of stresses thus playing a role in plant defense. Biotic elicitors are complex chemicals produced in living organisms with unknown chemical composition and include cell wall components of microorganisms and extracts of yeast cells

ABIOTIC ELICITORS

Very few studies have been carried out regarding elicitation of plant cell cultures for secondary metabolite synthesis using abiotic elicitors. Abiotic elicitors also termed as stress agents are non-biological materials and include different types of inorganic salts, heavy metal salts (Cu and Cd ions) and physical factors like UV radiation, osmotic, salinity, drought, temperature, Ca^{2+}, high pH and other chemicals with different mechanisms of action. Recently ozone has also been declared as a fungal elicitor. Other examples of abiotic elicitors include osmotic agents, drought, salinity, thermal and hormonal elicitors

TISSUE CULTURE

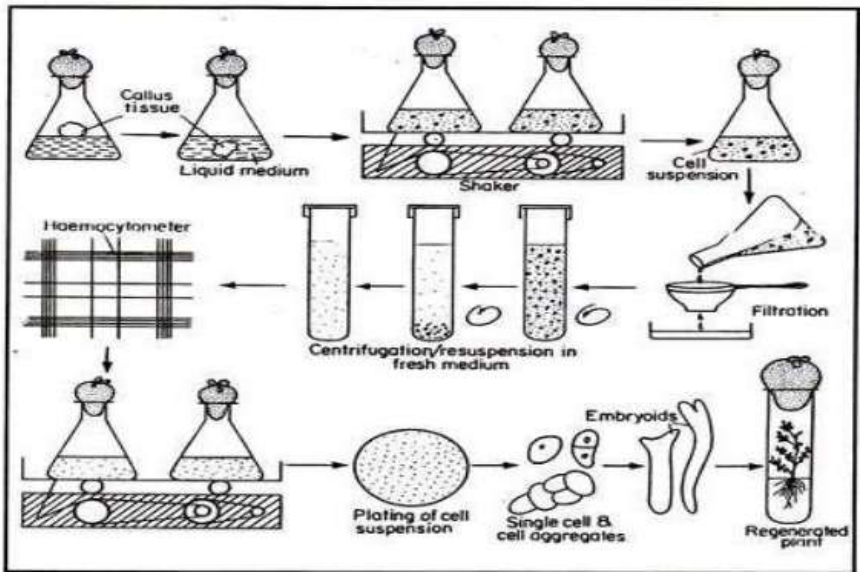
Production of secondary metabolites is under strict regulation in plant cells, due to coordinate control of the biosynthetic genes by transcription factors. Transcription factors are involved in secondary metabolism and their role has been reviewed by Endt *et al.* [31]. Several transcription factors involved in the regulation of alkaloid biosynthesis genes have been isolated and studied. There are indications that the abundance and activities of transcription factors per se are regulated by external signals [31, 32]. Based on this success, genetic transformation of medicinal plants has been attempted, primarily to enhance the production of various pharmaceuticals, but also flavors and pigments. The potential of metabolically engineered plant-derived secondary metabolites is high and has been well documented by modifying anthocyanin and flavonoid pathways, leading to changes in flower color, or increased levels of antioxidative flavonol production in tomato [33]. To date, however, there has been little success in modifying pathways to form pharmaceutically important compounds. Transgenic cultures and plants have been reported some time ago for about 70 species [34]. Hashimoto *et al*. [35] reported increased production of tropane alkaloids in genetically engineered root cultures. There are several strategies that can be used to enhance the production of desired pharmaceuticals by genetic engineering [36]. Oksman-Caldentey and Inzé [37] have reviewed the work on the production of designer metabolites in the post-genomic domain.

2. CELL SUSPENSION CULTURE

Cell suspension cultures are produced *in vitro* when friable calli are developed on liquid media in specially designed container and continuously shaken to give suspension of free cells. The medium is supplemented with plant hormones and maintained at specific conditions of temperature (25 to 28°C) and illumination (continuous dark or light, or photoperiod). The cultures are continuously agitated using a rotary shaker for a continuous supply of nutrients and oxygen to the growing cells. Conical flasks are used for this purpose due to the large surface area which can easily maintain a liquid medium and ensures continuous gaseous exchange. The suspension cultures may vary from large aggregates to finely dispersed cells. In case of aggregate formation, the size of aggregate depends upon the time for culturing, the composition of plant hormones and the plant species used.

There are two types of suspension cultures: the batch suspension cultures and continuous cultures. In the first type of suspension culture a part of initial cell suspension is sub-cultured using fresh media after regular time period. In the second type the fresh medium is added to the old culture medium after removal of excessive cell suspension after specific time period. These cultures are commonly used for commercial scale production of secondary plant metabolites. To maintain continuous cultures on large scale bioreactors like Chemostat are used.

During sub-culturing, the cells growing in suspension cultures may undergo changes in number of chromosomes and their effect on *in vitro* biosynthesis of secondary metabolites. A number of factors critically influence the optimization of secondary metabolite synthesis in culture. These factors include physico-chemical (for example illumination and inoculum size), nutritional (concentration of nitrate/ammonia, phosphate ions etc.) and hormonal factors (including concentration and type of growth regulators). Although a large number of issues are still needed to be resolved for commercial application of plant cell culture techniques for production of bioactive plant metabolites at large scale, extensive research is still going on and a few industrial processes are being performed using these cell suspension cultures.



3. BIOREACTOR BASED PRODUCTION OF VALUABLE SECONDARY METABOLITES

Plant cell culture technology, based on bioreactor cultivation of elite species has gradually become an efficient system for propagation of a wide range agricultural, forest, wild and medicinal plants species (Ziv, 2005, 2010; Businge et al., 2017). Plant biotechnology has also been recognized as an alternative source for the production of valuable secondary metabolites

Due to the specific morphology of the plants developing in vitro, the accessibility of nutrient during their cultivation in bioreactors is one of the primary factors, influencing both their development and their secondary metabolism (Steingroewer et al., 2013). Biomass accumulation and metabolite biosynthesis are often two-stage events, which require the development of a two-step cultivation strategy. In that cultivation

scenario, the factors that control the growth and multiplication of cultured cells/organs and biomass accumulation differs from the stimuli that assist the biosynthesis of secondary metabolites. Under these circumstances, the typical two-steps cultivation process requires the control and monitoring of different parameters on each stage. In the first stage – selection of high-producing cells or organ clones, the optimization of the suitable medium (salts, sugar, nitrogen and phosphate sources), plant growth regulator levels, and physical factors such as temperature, illumination, light quality, pH, agitation, aeration, and environmental gas (oxygen, carbon dioxide, and

ethylene) play critical role. Elicitation, replenishment of nutrient and precursor feeding, permeabilization and product recovery strategies assist with the accumulation of secondary

metabolites and have an impact in the second stage of the culture process. Following the two-steps specific strategy, it is possible to produce large amounts of biomass with an increase in the accumulation of secondary compounds (Murthy et al., 2014).

Bioreactors used for hairy roots are classified commonly into three types – liquid phase, gas-phase and mixed, which is a combination of both. Recently, the temporary immersion systems have been demonstrated to be very appropriate for cultivation of hairy roots, and production of valuable plant-derived secondary metabolites (Georgiev et al., 2012; Steingroewer et al., 2013; Georgiev et al., 2014). An important conclusion is that in bioreactor cultivation of plant cell suspensions, usually specialized cells in distinct developmental stages produce secondary metabolites (Balandrin et al., 1985). When the cells are undifferentiated, they wholly or partially lose their biosynthetic ability to accumulate secondary products (Rokem et al., 1985). Contrary, the hairy roots have attractive properties for secondary

metabolite production because of their differentiation, which is the same as the root system of intact plants, and faster growth in lack of hormonal treatment than plant cell cultures. Another advantage of hairy roots is that they often exhibit similar or greater biosynthetic capacity for secondary metabolite production compared to their mother plants (Banerjee et al., 1998; Kim et al., 2002).

The quality and quantity of active substances production from wild or field grown plants varied depending on the specific environmental conditions. With the in vitro cultivated plants in bioreactors, such problems could be avoided by the precisely controlled microenvironment. Studies of selected plant species prove that the bioreactor technologies allow obtaining of new pharmaceutically active compounds not existed in the field growing plants (Pavlov et al., 2005; Georgiev et al., 2010; Georgiev et al., 2014).

4. FERMENTOR TECHNOLOGY

Secondary metabolites branch out from the pathways of primary metabolism. Commercially, important secondary and primary metabolic pathways are given in Table

S. No.	Intermediates from primary metabolic pathway	Secondary metabolites derived
1.	Shikimic acid	Ergot alkaloids, antibiotics: candicidin and chloramphenicol
2.	Amino acids	Antibiotics: penicillin, cephalosporins and cephamycins, and gramicidin, immunosuppressive cyclosporine
3.	Acetyl-CoA and other Kreb's cycle intermediates	Antibiotics: erythromycin, antiparasitic avermectin antitumor doxorubicin, taxol
4.	Sugars	Antibiotics: streptomycin and kanamycin.

LIQUID FERMENTATION

Batch or fed-batch culture in submerged fermentation is employed for production of secondary metabolites. Inoculum is developed after careful strain improvement of producing organism. Initially, shake flasks culture is employed, and the culture which are in active growth phase are transferred to a small fermenter and later into a larger fermenter with production medium. Several parameters, like medium composition, pH, temperature, and agitation and aeration rate, are controlled. An inducer such as methionine is added to cephalosporin fermentations, phosphate is restricted in chlortetracycline fermentation, and glucose is avoided in penicillin or erythromycin fermentation.

SOLID-STATE FERMENTATION

Solid-state fermentation, defined as a microbial culture that develops on the surface and at the interior of a solid matrix and in the absence of free water, holds an important potential for the production of secondary metabolites. Two types of SSF can be distinguished, depending on the nature of solid phase used:

(a) solid culture of one support-substrate phase solid phase and

(b) solid culture of two substrate-support phase solid phase.

The **advantages** of solid-state fermentation in relation with submerged fermentation include: energy requirements of the process are relatively low, since oxygen is transferred directly to the microorganism. Secondary metabolites are often produced in much higher yields, often in shorter times, and often sterile conditions are not required.

It is important here to note our own experience of deriving actinobacterial secondary metabolite. Actinobacteria from terrestrial and marine habitats were screened for their antimicrobial activity. The bioactive metabolites were extracted and purified by thin layer and column chromatography, and the structure of the metabolite was elucidated by UV-spectrometry, FT-IR, mass spectrum analysis, and NMR. The derived metabolites staurosporine, octa-valinomycin, methyl-4,8-dimethylundecanate, and N-isopentyltridecanamide are known for their biological activity

5. SECONDARY METABOLITE PRODUCTION IN GENETICALLY ENGINEERED PLANT CELL CULTURE

The application of genetic engineering to produce genetically modified plants is currently one of the most significant molecular techniques in modern system of crop breeding. Considerable progress has been carried out in developing new more effective methods for transformation of various plants. *Agrobacterium* is one of the leading microorganism used for this purpose. Particularly, advancement in *Agrobacterium* - mediated genetic transformation of different types of agricultural crops such as legumes, cereals and other crop plants has resulted in development of transformation - enabling techniques, such as gene targeting, production of marker genes, and chromosomal engineering. These developments have significantly enhanced the efficiency of agricultural biotechnology by boosting the conventional methods of plant breeding to get high yield and better quality of crops. This has helped to raise the production of economically important crops to meet their commercial supply. Agricultural biotechnology has also helped in reducing crop losses due to diseases and pest attack, has increased the nutrient utilizing potential of food and animal feeds, has increased shelf life of fruits and vegetables thereby reducing post harvest losses, and has increased the resistance level of crop plants against stress making them tolerant to various environmental stresses such as drought and cold.

AGROBACTERIUM TUMEFACIENS-MEDIATED TRANSFORMATION

The term agricultural biotechnology refers to any method that changes the organisms or their parts to produce or modify agricultural products, thereby improving the quality of crops, or to produce microorganisms for particular uses in agricultural practices. Making it simpler, the application of biotechnological techniques to agriculture is termed as "agricultural biotechnology". In today's world genetic engineering comprises more than a half of agricultural biotechnology. Currently it is an important tool to perform genetic

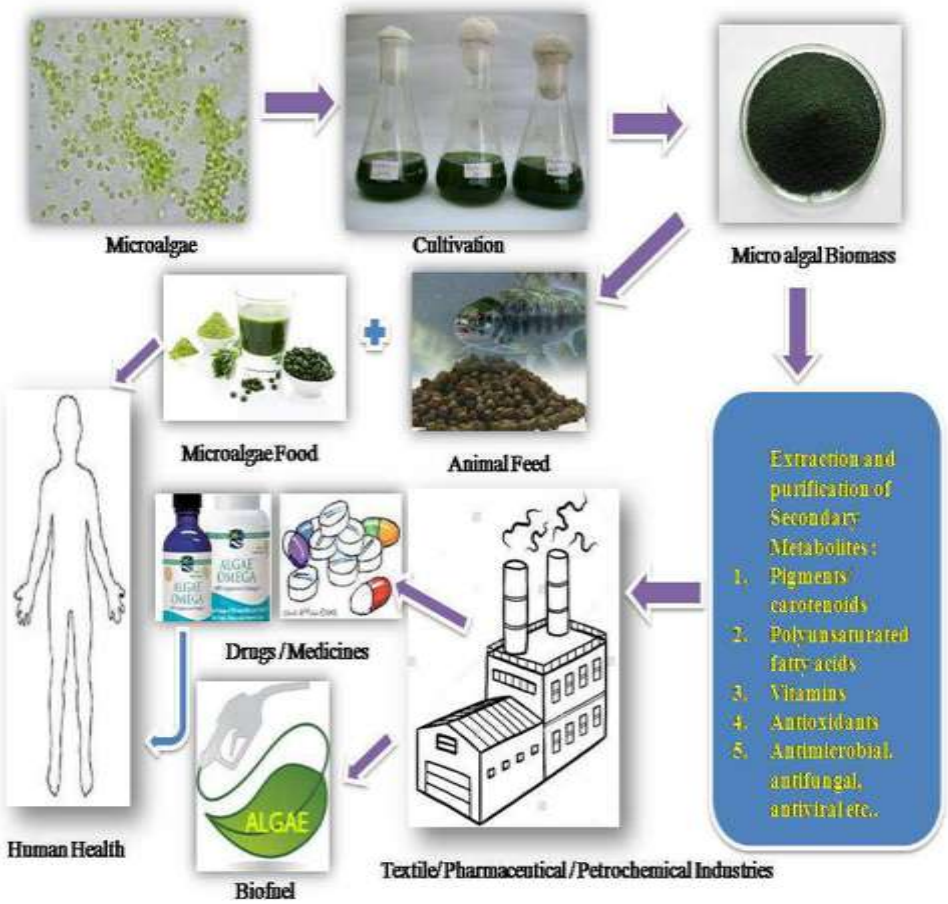
transformation and modification applicable to almost any plant species, including the major agriculture crops. Genetic transformation, *i.e.*, the insertion of foreign genes into the genome or genetic structure of plants, is the most commonly applied method in every technique implicated in agricultural biotechnology. The two commonly used methods for genetic transformation of plants are *Agrobacterium*-mediated transformation in which the naturally occurring soil bacterium is used for genetic modification of target plants; and Biolistic Transformation that involves the use of mechanical methods. In both of these methods, the gene of interest is inserted into the genome a specific plant followed by conventional breeding systems to transfer the newly introduced trait into other crops.

Genetic transformation is currently used as an efficient method for increasing the secondary metabolites production that have pharmaceutical values. The method is one of the most important biotechnological methods used to control the secondary metabolites synthesis in *in vitro* cultures. The most significant advancement and achievement of genetic transformation is considered to be the *in vitro* regeneration of medicinal plants using different explants to increase the synthesis of secondary metabolites leading to the manufacture of high-quality medicines that are plant based.

DNA technology is also helping a lot to boost and improve research related to the biosynthesis of secondary metabolite. The research related to this issue has been largely focused on identification of enzymes regulating a particular metabolic pathway and then modify the activities of these enzymes to modify the biosynthetic pathway. Transformation technology has been used for the genetic modification of almost 120 species belonging to 35 plant families, including many economically important crops, medicinal, ornamental, fruit, vegetables, tree and pasture plants, either by using direct or *Agrobacterium* mediated transformation methods. The transformation using *Agrobacterium* has many advantages than methods involving direct gene transfer. For example it is possible to transfer only a single or limited number of copies of DNA segments having the genes of choice at high rate with low cost or the transfer of large DNA segments with almost no rearrangements. The ultimate goal of all these practices is the high yield of a targeted compound, and at the same time reducing the cost as compared to the natural synthesis of that metabolite by the plants.

Agrobacterium is a group of widespread naturally occurring soil borne bacteria that infect plants. Infections are caused by the transfer of bacterial genome into the host cells at the point of infection. This bacterial genome then integrates into the genome of host cell. The integrated genetic material is called transferred DNA (T-DNA) that is a part of a large

plasmid. A plasmid is a small circular DNA present universally in all bacteria. The inherent ability of these microbes to modify the genetic composition of any plant laid the basis for genetic modification of plants by using *Agrobacterium*. At present, genetic transformation using *Agrobacterium* is the most widely used procedure for genetic engineering in plants due to its high efficiency. Initially, it was thought that the bacterium only can infect dicot plants, but later it was observed that it can also transform monocot plants for example rice (ABNE, 2010). The genus *Agrobacterium* has been divided into different species on the basis of disease symptoms and host range. Among different species *A. radiobcater* is an "avirulent" species which does not cause any infection. *A. rhizogenes* causes hairy root disease. *A. tumefaciens* is the causal organism for crown gall disease in plants. The *A. rubi* causes cane gall disease while *A. vitis* infects grapevines (Rout, 2001). Strains of *A. rhizogenes* and *A. tumefaciens* are characterized by the presence of either a Ri or Ti-plasmid, while the strains of *A. vitis* usually have a Ti-plasmid.

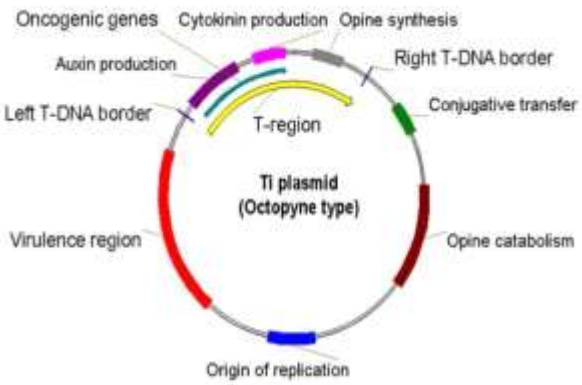


AGROBACTERIUM TUMEFACIENS

A. tumefaciens is a soil-borne plant pathogenic bacterium that causes tumor formation (crown galls) in plants that are infected by this bacterium. These tumors are characterized by the presence of specific low molecular weight compounds called opines. The bacterium uses these opines as source of nitrogen and energy which are specific for a specific *Agrobacterium* strain. There are about 30 different opines identified so far.

A. tumefaciens is characterized by the presence of large plasmids the Ti plasmid that is transferred to the infected plant at the site of infection. A specific portion of this plasmid (T-DNA) is incorporated into the host cell genome that induces the formation of tumor. The T-DNA is involved in the synthesis of opines - the carbon and nitrogen containing compounds that are essential for the continuous growth of the bacterium. There are three different classes of opines, the octopine, agropine and nopaline. *A. tumefaciens* can infect more than 600 species belonging to about 330 genera. The development of infection varied depending upon the variety of plant species and the type of plant organ that is infected. In general the monocots except for some species of Arales and Liliales are not susceptible to this bacterium.

A. tumefaciens causes genetic transformation of its host by integration of a selected DNA fragment from its Ti plasmid to the chromosome of host-cell. This integrated DNA segment (T-DNA) has a group of opine-catabolism genes and oncogenes that cause neoplastic growth of the tissue in which they are transferred and produce opines. Opines are derived from amino acids that are a source of nitrogen for many bacteria. The transfer of T-DNA is not sequence specific. This factor makes it possible to replace the native T-DNA genes with any required gene(s) to be inserted in target plant and its delivery into the host cells (Figure 1).



Structure of Ti plasmid

Genetic transformation using *A. tumefaciens* has been done in various medicinal plants. The process has been used for successful transformation of *Azadirachta indica* (neem)

regenerated *in vitro* using *A. tumefaciens* having a recombinant plasmid. Similarly *A. belladonna* was genetically modified using *A. tumefaciens,* to improve its alkaloid composition. Genetic transformation of *Echinacea purpurea* using *Agrobacterium* has also been reported using leaves as explants.

6. HAIRY ROOT CULTURES

Hairy roots are specific cultures of genetically modified roots produced by the higher plants infected with *Agrobacterium rhizogenes*. This bacterial species causes the hairy root disease causing the neoplastic development of roots that have abnormally high development rate in media free of any type of growth hormone and are genetically stable. These roots essentially synthesize the same compounds as are produced in wild type species. High yield and stability of these roots make them an important source to produce bioactive plant secondary metabolites. The synthesis and accumulation of these compounds can further be enhanced and small and large scale by using specific elicitors. At larger scale bioreactors are used for optimum metabolite production. Hairy roots can be used as natural source for the synthesis of recombinant proteins, hence are also important in related industries as well. An understanding of the underlying molecular mechanisms which lead towards their formation have helped in improving this technique.

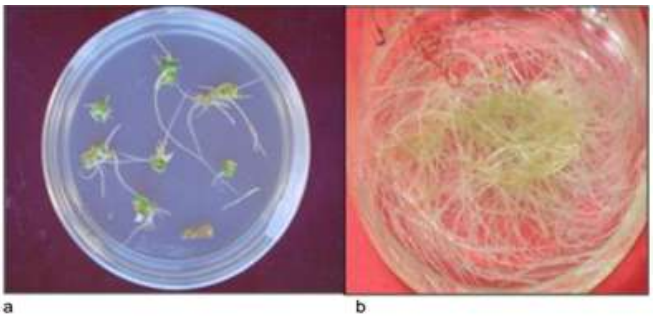
AGROBACTERIUM RHIZOGENES

A. rhizogenes - a Gram negative bacterium is soil-borne microorganism and belongs to the family *Rhizobiaceae.* The bacterium causes a disease called hairy roots by causing infection in wounded plant species. The disease develops when the pathogen transfers a large sized plasmid called root-inducing (RI) plasmid into the genetic material of the plant that is infected by this bacterium. This plasmid carries a specific DNA portion (T-DNA) that is flanked by 25 bp oligonucleotide repeats. This T-DNA actually causes the disease in the infected plants. This T-DNA has a set of specific genes that code for the enzymes that control the biosynthesis of phytohormones cytokinin and auxin and some unusual amino acids such as opines. The production of these enzymes changes the hormonal balance that induces the production of specific roots at the site of infection which proliferate at a very high rate and are called hairy roots. These hairy roots are characterized by rapid growth that is not dependent on the presence of hormones, do not show geotropism, produce lateral branches and are genetic stable. For the last several decades their stable nature and high rate of

production have made hairy root cultures a valuable source for the *in vitro* biosynthesis of bioactive metabolites of plant origin especially those that are produced in intact roots.

Stable hairy root cultures for more than 200 plant species have been prepared that are able to produce a large number of distinct secondary plant metabolites and their success is largely due to their ability to optimize their metabolic functions in response to various abiotic and biotic stimuli. Recent advances in technology have helped to understand the underlying mechanisms at molecular level that are involved in the transfer of T-DNA into the target plants. The mechanism of action of *A. rhizogenes* is principally similar to that of *A. tumefaciens.*

The hairy roots formed as a result of infection by *A. rhizogenes* usually have a very high growth rate and are genetically stable. In some plant species hairy roots can be propagated to produce whole plants. *A. rhizogenes* mediated transformation has been applied to get transgenic plants in 89 plant taxa for 79 different species belonging to 55 genera and 21 families of higher plants. All these plants possess a specific phenotype characterized by reduced apical dominance in roots and stems, a condition called hairy root syndrome, short internodes, wrinkled leaves with high width to length ratio, high rate of root growth in culture, plagiotropic roots behaving differently towards geotropism, changes in flower structure, delay in flowering, reduction in pollen and seed production ultimately causing reduction in fertility.



Hairy root culture in *Tagetes erecta* L. a) Hairy root induction from leaf discs infected with *A. rhizogenes*, b) Growth of isolated rhizoclone in liquid MS media.

7. FACTORS EFFECTING LARGE SCALE PRODUCTION OF SECONDARY METABOLITES

- **NITROGEN.**

Plant tissue culture media, such as MS, LS, or B5, have both nitrate (NO–3) and ammonium (NH+4) as sources of nitrogen. For example, nitrogen source is very important for plant suspension cultures of *Holarrhena antidysenterica* for accumulation of alkaloids [65], in cell suspensions of *Vitis vinifera* for anthocyanin formation, and in shikonin production by *Lithospermum erythrorhizon* cell cultures [25]. The ratio of NH+4 /NO–3 and overall levels of total nitrogen have been shown to markedly affect the production of secondary plant products. The reduced levels of NH+4 and increased levels of NO–3 promoted the production of shikonin and betacyanins, whereas higher ratios of NH+4 to NO–3 increased the production of berberine and ubiquinone [14]. Reduced levels of total nitrogen improved the production of capsaicin in *Capsicum frutescens*, anthraquinones in *Morinda citrifolia*, and anthocyanins in *Vitis* species [54, 64].

PHOSPHATE

Its concentration in the medium has a great effect on the production of secondary metabolites in plant cell cultures. Higher level of phosphate enhanced the cell growth, whereas it had a negative influence on secondary product accumulation. Medium limited in phosphate either induces or stimulates both the product and the levels of key enzymes leading to the product. Reduced phosphate levels induced the production of ajmalicine and phenolics in *Catharanthus roseus* and nicotin in *Nicotiana tabacum* [35]. In contrast, increased phosphate was shown to stimulate synthesis of digitoxin in *Digitalis purpurea* and betacyanin in *Chenopodium rubrum* [5].

POTASSIUM ION (K+)

It serves as a major contributor to osmotic potential, a specific requirement for protein synthesis, and an activator for particular enzyme systems [65]. Higher K+ concentration caused slower cell growth. More of soluble sugar was stored within the cells under K+ deficiency.

MICROELEMENTS

are required in trace amounts for plant growth and development, and have many diverse roles [21]. Manganese, iodine, copper, cobalt, boron, molybdenum, iron, and zinc usually comprise the microelements, although other elements, such as nickel and aluminum, are frequently found in some formulations. Iron is usually added as iron sulphate, although iron citrate can

also be used. Ethylenediaminetetraacetic acid (EDTA) is used in conjunction with the iron sulphate. EDTA complexes allow slow and continuous release of iron into the medium. Uncomplexed iron can precipitate out of the medium as ferric oxide. Plant cell cultures are usually grown heterotrophically using simple sugars as carbon source and inorganic supply of other nutrients. The level of sucrose affected the productivity of secondary metabolites in cultures [35].

SUCROSE OR GLUCOSE

They are at 2 to 4% are suitable carbon sources, which are added to the basal medium. Fructose, maltose, and other sugars also support the growth of various plant cells. The choice of the most suitable carbon source and its optimal concentration depend on the plant species and products.

VITAMINS

Such as thiamine (vitamin B1) and myo-inositol, are considered essential for the culture of plant cells in vitro. However, other vitamins are often added to plant cell culture media. Thus, MS medium includes myoinositol, nicotinic acid, pyridoxine HCl, and thiamine HCl [14].

AMINO ACIDS

They are also commonly included in the organic supplement. The most frequent are glycine (arginine, asparagine, aspartic acid, alanine, glutamic acid, glutamine, and proline are also used), but in many cases their inclusion is not essential [21]. Amino acids provide a source of NO_3^- and, like NH_4^+ , uptake causes acidification of the medium. Other supplements include casamino acid, peptone, yeast extracts, malt extracts, and coconut milk. Coconut milk is also known as a supplier of growth regulators.

GELLING AGENTS

Media for plant cell culture in vitro can be used in either liquid or solid forms, depending on the type of culture being grown. For any culture types that require to be grown on the surface of the medium, it must be solidified or gelled. Agar, produced from seaweed, is the most common type of gelling agent, and is ideal for routine applications. However, because it is a natural product, the agar quality can vary from supplier to supplier and from batch to batch.

GROWTH REGULATORS

Concentration is often a crucial factor in secondary product accumulation [63]. Phytohormones or growth regulators are required to induce callus tissues and to promote the growth of many cell lines. Since each plant species requires different kinds and levels of phytohormones for callus induction, its growth, and metabolites production, it is important to select the most appropriate growth regulators and to determine their optimal concentrations.