CHAPTER I
INTRODUCTION

1.1 The importance of the plant kingdom

The plant kingdom has provided a wide variety of natural products with diverse chemical structures and a vast array of biological activities, many of which have found applications in the health sciences. For centuries, plants have been an important source of precursors and products used in a variety of industries including those dealing with pharmaceuticals, food, cosmetics and agriculture (Primrose, 1987). During the past decade, there has been considerable demand for plant based natural products as opposed to the synthetically product. This is because of wide spread awareness regarding the harmful effect associated with synthetic chemicals (Bagde *et al.*, 1994).

Recently, the raw materials from natural products become limited in supply. Also, their availability and quality may vary. The disadvantage of this situation and the need for large volume of naturally derived materials makes biotechnology, an effective alternative, to salvage traditional natural raw materials availability. Biotechnology has contributed significantly towards the procurement of natural products (Bagde *et al.*, 1994). The *in vitro* culture of plant cells and tissues is one of the growth areas of biotechnology because of its potential to generate improved crops and ornamental plants (Primrose, 1987). Moreover, plant cell cultures may become alternative sources for higher plant products which can not be chemically synthesised.

1.2 Plant cell and tissue cultures

Plant cell and tissue culture method has gained considerable importance towards the production of biologically active complex organic molecules.

By 1950, a methodology had been developed to allow culture of numerous species and manipulation of the growth medium could lead to initially formed, largely-homogeneous tissue developing roots, shoots, or indeed regenerating a plantlet. This resulted in the commercial development of micropropagation of agricultural and horticultural species as well as providing relatively undifferentiated biomass for studies on plant physiology. By 1970, reports of secondary metabolism in plant cell and tissue cultures had appeared, and the subject has burgeoned with the

idea 50 that plant cells could be manipulated like microorganisms to accumulate metabolites in yields akin or superior to those from field-grown plants (Banthorpe, 1994).

The advantages of plant cell cultures over living plants, in terms of secondary metabolite production, are clear:

a. Growth conditions are laboratory controlled therefore reproducible yields of end product are achieved;
b. Growth parameters such as pH, changes in nutrient media, temperature, etc. and be optimised to achieve metabolite production in yield significantly higher than in the living plants;
c. Separation of target compounds is much easier due to lower complexity of extract;
d. Plant cell cultures are an excellent source of enzymes, much superior to living plants where isolation often leads to enzyme denaturation.

With enzyme availability, the opportunity to perform biosynthetic and/or biotransformation experiments related to metabolite production, is clear (Kutney, 1999).

Plant cell and tissue culture is considered a viable alternative for production, if the product formation rate, yield, and final concentration are increased significantly. For successfully, these following strategies should be expanded to include an induction step (Heinstein, 1985)

1. Selection of a high-yielding plant,
2. Establishment of cell cultures from the selected plant,
3. Development of an optimum growth medium, without consideration of secondary natural product production,
4. Development of methods to induce secondary natural product formation,
5. Clonal selection of highly inducible cell strains, and
6. Development of optimum production medium.

1.3 History and perspective

The ability of an individual cell to grow and divide in a self-regulating manner was first expanded in the cell theory of Schliden (in 1838), and Schwann (in 1839), the latter of which also incorporated the concept of totipotency. Totipotency means a state of undifferentiation in plant cells, whereby they possess all the genetic material

required to demonstrate any characteristic of the living plant. The techniques of plant cell and tissue culture have been developed since 1902 when Haberlandt was first successful in cultivating single plant cells in simple nutrient solution. How he did not observe major cellular growth or cell division. In the 1930's the first *in vitro* cultures were established by White (in 1934) and Gautheret (in 1939), and Routien and Nickjel used the concept of plant cell cultures to produce various products in 1956 (Street, 1977). Following this, more and more plant species were introduced into plant cell culture. In 1962, Murashige and Skoog developed a chemically well defined growth medium which offered the prospect for the use of plant cells for secondary metabolite production (Murashige and Skoog, 1962). The 1970's were marked by developments in large scale growth and process technology for cell culture. In 1982, at least 30 compounds were known to accumulate in plant culture systems in concentrations equal to or higher than that of the intact plant. The techniques were combined with recent advances in developmental, cellular and molecular genetics, and using conventional plant breeding have turned plant biotechnology into an exciting research field. These have become major tools in the study of an increasing number of fundamental and applied problems in the plant sciences. These expanding uses to investigate cell and developmental biology, biochemistry physiology, genetics, and molecular biology are providing new knowledge about fundamental characteristics of plants. They provide a vital tool for biosynthesis and biotransformation studies of different chemical nature, and these have been broadly documented. Now, the commercial interest for large-scale production of plant cell cultures is continuing increasingly.

In essence, plant cell culture technology is recent innovation which has progressed rapidly in the last 30 years and is now recognized as a major discipline, playing an equally important role in elemental biological studies and product oriental research.

1.4 Definition

Plant tissue culture is the technique of growing plant cells, tissues, and organs in a prepared suitable nutrient medium under the sterile conditions. The process is whereby small pieces of the living tissue (explants) are isolated from organism and grown aseptically for indefinite periods on nutrient medium. For successful plant tissue culture, it is best to start with an explant rich in undetermined cells, e.g. those

from the cortex of meristem, because such cells are capable of rapid proliferation. The usual explants are buds, root tips, nodal segments or germinating seeds and these are placed on suitable culture media where they grow into an undifferentiated mass known as a callus (Primrose, 1987).

1.5 Callus culture

A callus consists of an amorphous mass of loosely arranged thin-walled parenchyma cells arising from the proliferating cells of the parent tissue. Frequently, as a result of wounding (injury), a callus is formed at the cut end of a stem or root (Dodds and Roberts, 1985). Callus culture is obtained from any part of plant, both from plant organs and from specific tissues or cells (Collin and Edward, 1998).

The initiation of callus is referred to callus induction. The most commonly used starting material wase seedling, which was germinated from surface sterilised seeds, or explants. They are transferred to semi-solid media by aseptically techniques. Maintain once of callus culture is carried out by keeping them at 25 ± 2 °C with light and routine subculture to new media every 4-8 weeks, depending on the individual cell growth.

1.6 Cell suspension culture

Suspension culture is obtained from callus culture. It can be grown in bulk, show a faster growth rate than callus culture and cells are exposed uniformly to the media. Cell suspension provides a source for large scale embryogenesis and for commercial production of secondary metabolites.

The initiation of suspension culture is made by transferring callus into a liquid media with the same ingredients as that used in callus, and agitating the cell mass breaks up to give isolated cells, small clusters of cells and much larger aggregates. After shaking, the suspension of cells consists of cell aggregates of varying size and cell number, and single cells. Maintain once of suspension cultures is carried out by keeping them on an orbital shaker (50-130 rpm.) with the same conditions as callus culture and routine subculture to new media every 2-4 weeks, depending on the individual cell growth.

Several forms of suspension culture are commonly utilised, as follows, (Evan, 1996)

1. Batch suspension cultures

In this technique the cells multiply in a liquid medium which is being continuously agitated to break up any cell aggregates. Except for the circulation of air, the system is 'closed' with respect to additions or subtraction from the culture. Typically, the original inoculation of cells into the medium is followed by a lag period and then, after increasing in mass, the cells undergo a period of exponential growth and division. Finally, a stationary growth phase is reached, at which point some component of the medium, essential for growth, has probably been exhausted. Growth will recommence when cells are transferred to a fresh medium or when more medium is added to the original culture.

2. Semicontinuous cultures

In this instance the system, an 'open' one, is designed for the periodic removal of culture and the addition of fresh medium, by which means the growth of the culture is continuously maintained.

3. Continuous cultures

Two forms of this 'open' system are the chemostat and turbostat systems, in which the volume of culture remains constant and fresh medium and culture are, respectively, continuously added and withdrawn. The essential feature of these two systems is that cell proliferation takes place under constant conditions. In the chemostat arrangement, a steady state is achieved by adding medium in which a single nutrient has been adjusted so as to be *growth-limiting*; this contrasts with the batch culture method, in which the transient conditions in which the cells find themselves lead to continuous changes in their growth rate and metabolism.

1.7 The requirements of cultures

In order to control both physical and chemical environments of plant cell cultures, aseptic conditions must be established, to avoid any contamination. The basic requirements for plant cell culture work are (Dixon and Franklin, 1985):

1. An area for medium preparation
2. A sterile room or sterile air cabinet for aseptic transfer
3. A constant temperature room or incubator for growth of callus cultures
4. Shaker facilities for cell suspension cultures.

Plant cell and tissue cultures grow differently depending on the type of culture condition they are subjected to. There are several factors influencing the cell growth and secondary metabolites production, such as culture media and external environment condition.

1.8 Culture media

Culture media might be satisfied as internal environment condition or chemical factors. The selection or development of the culture medium is vital to success in tissue culture. No single medium will support the growth of all cells, and changes in the medium are often necessary for different types of growth response in a single explant. A literature search is useful for selecting the appropriate medium. If literature on the plant is not available, the development of suitable medium is based on trial and error.

Culture media is including media compositions and plant growth regulators. In general, components of typical plant tissue culture media include (Brownleader and Dey, 1997);

1. Inorganic macronutrients (e.g. Fe, Mg, Ca, K, P and N)
2. Inorganic micronutrients (e.g. Mn, Cu, Zn, B, Na, Cl, I, S, Mo, Co, Al and Ni)
3. Organic nitrogen sources (e.g. glycine and inositol)
4. Vitamins (e.g. nicotinic acid, pyridoxine and thiamine)
5. Carbon sources (e.g. glucose and sucrose)
6. Plant growth regulators (e.g. auxins and cytokinins)
7. Optional organic compound (e.g. casein hydrolysate and yeast extract)
8. Antibiotics such as kanamycin
9. A gelling agent (0.5-1.0% w/v good quality, bacteriological grade agar) if medium is to be semi-solid.

Culture media is one of important factor which must be optimised for the growth and maintenance of callus and suspension cultures. For example, levels of carbon source (Wang *et al.*, 2001), mineral nutrients (Do and Cormier, 1991), and plant growth regulators (O'Dowd *et al.*, 1993) are required in optimisation of medium (Murashige and Skoog, 1962, and Gamborg *et al.*, 1968) for increasing cell growth and secondary metabolite production (Hilton and Rhodes, 1994, Hilton and Wilson, 1995, and El Sayed and Verpoorte, 2002).

1.8.1 Media composition

The first decision to be made when initiating a plant callus culture is the composition of culture medium. The most commonly used media are Murashige and Skoog (MS) (Murashige and Skoog, 1962), Gamborg B5 (B5) (Gamborg *et al.*, 1968), and some other media modified from the above. These media include different amounts of inorganic nutrients, trace elements, iron source(s), a carbon source, and organic supplements. Many of the constituents or medium formulation influence culture initiation, growth rate, biomass formation, specific product synthesis, and accumulation of secondary metabolites as well. A variety of medium compositions have been shown to affect product yield, of which carbon source, nitrogen source, and phosphate level are most often studies.

1.8.2 Plant growth regulators (PGRs)

Plant growth regulators (PGRs), or plant hormones, or phytohormones are organic compounds naturally synthesised in higher plants, which influence growth and development. These compounds are specific in action, are active in very low concentrations, and regulate cell enlargement, cell division, cell differentiation, organogenesis, senescence and dormancy (Evan, 1996).

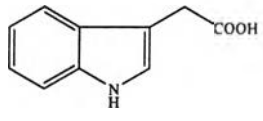
PGRs tend to be omitted from the basic medium formulation. They can be divided into 5 major groups; auxins, cytokinins, abscisic acid, gibberellins, and ethylene. The type and concentration of PGRs used will vary according to the cell culture purpose. Normally, the widely used PGRs are auxins and cytokinins, which help maintain dedifferentiated cell growth and promote cell division, respectively. The effects of auxins and cytokinins, and the balance between their concentrations play an important role in cell differentiation (Wilson *et al.*, 1991) and secondary metabolites production (Morris *et al.*, 1985). The appropriate level of auxins and cytokinins induce proliferative growth and callus formation, and plant regeneration from callus as well.

1.8.2.1 Auxins

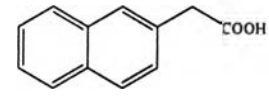
Auxins are a class of compounds that required by most plant cells for division and root initiation. At high concentrations, auxins can suppress morphogenesis. The most commonly used auxins and their molecular weights are listed in Table 1, and their chemical structures are shown in Figure 1.

Table 1 Common auxins used in plant cell cultures

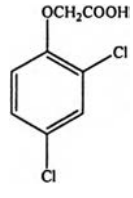
Auxins	Molecular weight
Indole-3-acetic acid (IAA)	175.2
α-Naphthaleneacetic acid (NAA)	186.2
2,4-Dichlorophenoxyacetic acid (2,4-D)	221.04
Indole-3-butyric acid (IBA)	203.2



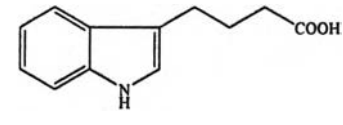
Indole-3-acetic acid (IAA)



α-Naphthaleneacetic acid (NAA)



2,4-Dichlorophenoxyacetic acid (2,4-D)



Indole-3-butyric acid (IBA)

Figure 1 Chemical structures of common auxins used in plant cell cultures

The auxin 2,4-D is widely used for callus induction whilst IAA, IBA, and NAA are used for root induction. Synthetic auxins such as 2,4-D and NAA can replace the naturally occurring auxin IAA because IAA is readily oxidised by plant cells. Normally, 2,4-D is included in many plant tissue culture media as it can stimulate both cell division and cell expansion in many systems (Dodds and Robert, 1985, Morris *et al.*, 1985, and Franklin and Dixon, 1994). Auxins also help in maintaining dedifferentiated cell growth (Franklin and Dixon, 1994). The requirement of auxins in cell culture is different; callus initiation in some plants need

less than 0.1 mg/l of 2,4-D whilst in other plants need up to 5-10 mg/l. Generally, callus growth may require lower levels of auxins than are needed for callus induction (Franklin and Dixon, 1994).

Auxin stocks are usually prepared by weighing out 10 mg of auxin into a 200-ml beaker, adding several drops of 1N NaOH or KOH until the crystals are dissolved (not more than 0.3 ml), rapidly adding 90 ml of double-distilled water, and increasing the volume to 100 ml in a volumetric flask. Make IAA stocks fresh weekly; IAA is degraded within a few days by light and within several hours to a few days by plant tissue.

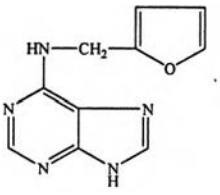
Auxins are thermostable. However, IAA is destroyed by low pH, light, oxygen and peroxidases (Posthumus, 1971); while NAA and 2,4-D are more stable forms of auxins.

1.8.2.2 Cytokinins

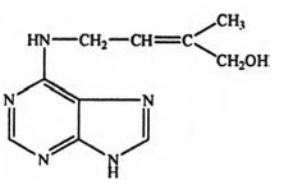
Cytokinins are a class of compounds that promote cell division, shoot proliferation, and shoot morphogenesis. The most commonly used cytokinins and their molecular weights are listed in Table 2, and their chemical structures are shown in Figure 2.

Table 2 Common cytokinins used in plant cell cultures

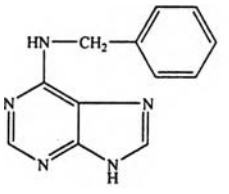
Cytokinins	Molecular weight
6-Furfurylaminopurine or kinetin (Kn)	215.2
6-Benzyl-aminopurine (BA)	225.2
$N^6(\Delta^2$-isopentenyl)-adenine(2iP)	203.3
trans-6-(4-hydroxy-3-methylbut-2-enyl) amino purine or zeatin (Zn)	219.2



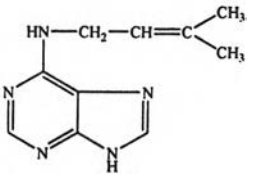
Kinetin (Kn)



Zeatin (Zn)



Benzyladenine (BA)



Isopentenyl adenine (2iP)

Figure 2 Chemical structures of common cytokinins used in plant cell cultures

Kinetin is the most widely used, it is typically added at 0.1 mg for callus induction, and it can be used higher to induce the rapid multiplication, shoot formation, axillary buds and meristems. Some substances also exhibit cytokinin-like action as well, such as diphenylurea (DPU), thidiazuron (TDZ) and a growth factor in coconut milk. Autoclaved coconut milk can be added to culture media as a cytokinin source at a concentration of 10-15% in the medium (Dodds and Robert, 1985, and Franklin and Dixon, 1994).

Cytokinin stocks are prepared in a fashion similar to that for auxin stocks, except that 1N HCl and a few drops of water are used to dissolve crystals. Gentle heating is usually required to completely dissolve crystals. Double-distilled water is rapidly added to avoid the crystals falling out of solution. Bring the stock up to the desired volume in a volumetric flask. Cytokinin stocks can be stored for several months in the refrigerator.

Cytokinins (Kn and Zn) are thermostable; no breakdown products were detected after 1 hr at 120 °C (Dekhuijzen, 1971); 2iP and BA are stable for 20 min at 100 °C.

1.9 External environment conditions

Plant cell cultures grow differently depending on the type of culture condition they are subjected to. The optimum environment conditions or physical factors are required for establishment and maintenance of the cell culture. These are composed

of temperature, light (intensity, type and duration), and pH of the medium which are varying depend on plant species. They are playing important role in morphogenesis of the culture and also related with increasing of total yield of secondary metabolites. For example, cultivation temperature is often maintained at around 25 ± 2°C, but some plant species may require varying temperature for optimum growth. Light might promote embryogenesis, shooting and greening of callus, and can be essential for production of secondary metabolites. Light is usually supplied by cool-white or special plant growth fluorescent lamps. Different light regimes have been reported to increase the accumulation of volatile constituents (Charlwood *et al.*, 1989, and Reil and Berger, 1997). In addition, pH of medium can influence the uptake of nutrients and precursors, the permeability of membranes, and release of secondary metabolites. Normally, pH of the medium is adjusted to 5.7-5.8 for optimum growth and accumulation of secondary metabolites.

For suspension cultures, the environmental conditions also including flask shaking rate. The shaker speed and the gaseous regime may also influence the growth and secondary metabolite formation.

1.10 Applications

Today, the plant cell and tissue culture, particularly modern plant biotechnology, is an important tool to produce plant specific products of high value for commercial purpose. It has also been widely used both basic and applied studied in plant sciences, with significantly impact on agriculture, horticulture, pharmaceutical, food, cosmetics and forestry. Research in plant cell and tissue cultures can be divided into 5 broad areas, namely: (Trevor, 1990)

1. Cell behaviour
2. Plant modification and improvement
3. Pathogen-free plants and germ storage
4. Clone propagation
5. Production of primary and secondary metabolites

Recently, the research in production of secondary metabolites has achieved great significance. Much of these are related to the potential for producing commercial products, especially in the food and pharmaceutical industries (Trevor, 1990).

1.11 Secondary metabolites

1.11.1 Definition

Secondary metabolites refer to any chemical compounds which are not essential for survival of plant cell, in contrast, where the *in vitro* role of these compounds has been ascertained, it is certain that they are often essential for the survival of plant as a whole. Secondary metabolites are often characteristic of a plant family. Difference in chemical structures of them and their site of locations in plants make it difficult to identify a common function for all secondary compounds. There is evidence for them having a very important role as constitutive compounds in the plant's defence against insect, pests, and predators, particularly in mature tissue that is accumulated these compounds. Secondary metabolites can also be synthesised rapidly in response to microbial infection and insect attack, and the same compounds appear to be synthesised rapidly in response to a range of physical stresses such as cold, heat, osmotic pressure and physical damage.

Synthesis of secondary products appears to be stimulated if fixed carbon is not fully utilised by the primary metabolic activities of cell growth and differentiation. Carbon not used to synthesise cell walls and protein is converted into secondary compounds and stored in the vacuole or cytoplasmic vesicles. When more rapid growth is resumed the secondary products are degraded and stored carbon released. The activities of secondary and primary metabolism are therefore closely related and exist in a dynamic equilibrium linked by key enzymes.

Secondary metabolites have provided an array of complex structures to identify and describe, however, because of the complexity of the biosynthetic pathways, knowledge of their synthesis has lagged behind. Current interest in manipulating the levels of these compounds in plants and tissue cultures has stimulated an increased interest in their synthesis.

Many of the plants which provide the herbs, spices, beverages, perfumes, essential oils and pharmaceuticals are grown in areas of the world where production may be limited by climatic conditions, or the unsophisticated nature of the agriculture. The possibility of an uncertain and variable supply of the products has prompted an examination of alternative and possibly more stable sources. One possibility was to treat plant cell suspensions in the same way as microbial-based fermentor systems and grow them on an industrial scale.

1.11.2 Biosynthetic pathway of secondary metabolites

Investigation of biosynthetic pathways of secondary metabolites involves the study of the mechanism of the synthesis of particular products. An important requirement for improvement of secondary metabolite synthesis is an understanding of the biosynthetic pathway and the enzymology of biosynthesis of particular products. In depth studies of pathway in whole plant are difficult, because the biosynthetic activities may be only declared in particular cell types within a specific plant organ or at a certain periods. In spite of the diversity of secondary metabolites, only a few intermediates used in primary metabolism provide the precursors for most secondary metabolites. The key intermediates are sugar, acetyl CoA, nucleotides, and amino acids. Many natural products are derived from pathway involving more than one of these intermediates. The main biosynthetic pathways are list in Fig. 1(Dörnenburg and Knorr, 1996). A number of techniques have been developed to investigate these diverse pathway and the sites of biosynthesis, including grafting, and the treatment of sterile tissue cultures or excised organs with radioactive precursors. Location of key secondary biosynthetic enzymes has in some aspects become the preferred method to achieve knowledge of the metabolic pathways.

1.11.3 Natural products

Particular natural products are often restricted to special plant families or genera. In many cases they are importance for the plant to survive in its environment. They have received a major interest because of their different functions and their impressive biological activities ranging from antimicrobial, antibiotic, insecticidal, and hormonal properties, to highly important pharmacological and pharmaceutical activities. Until now, most natural products used for this are still derived directly from plant material. Many plant used for the isolation of natural products have to be grown in the tropics or in subtropical regions, and collected from the wild. Also, some of these plants will be no longer available due to over collection. Plants are able to produce a considerable variety of phytochemicals with highly impressive biological activities. However, in many cases the limited supply of the drugs is a nearly insurmountable problem. The establishment of cell culture systems might overcome this problem.

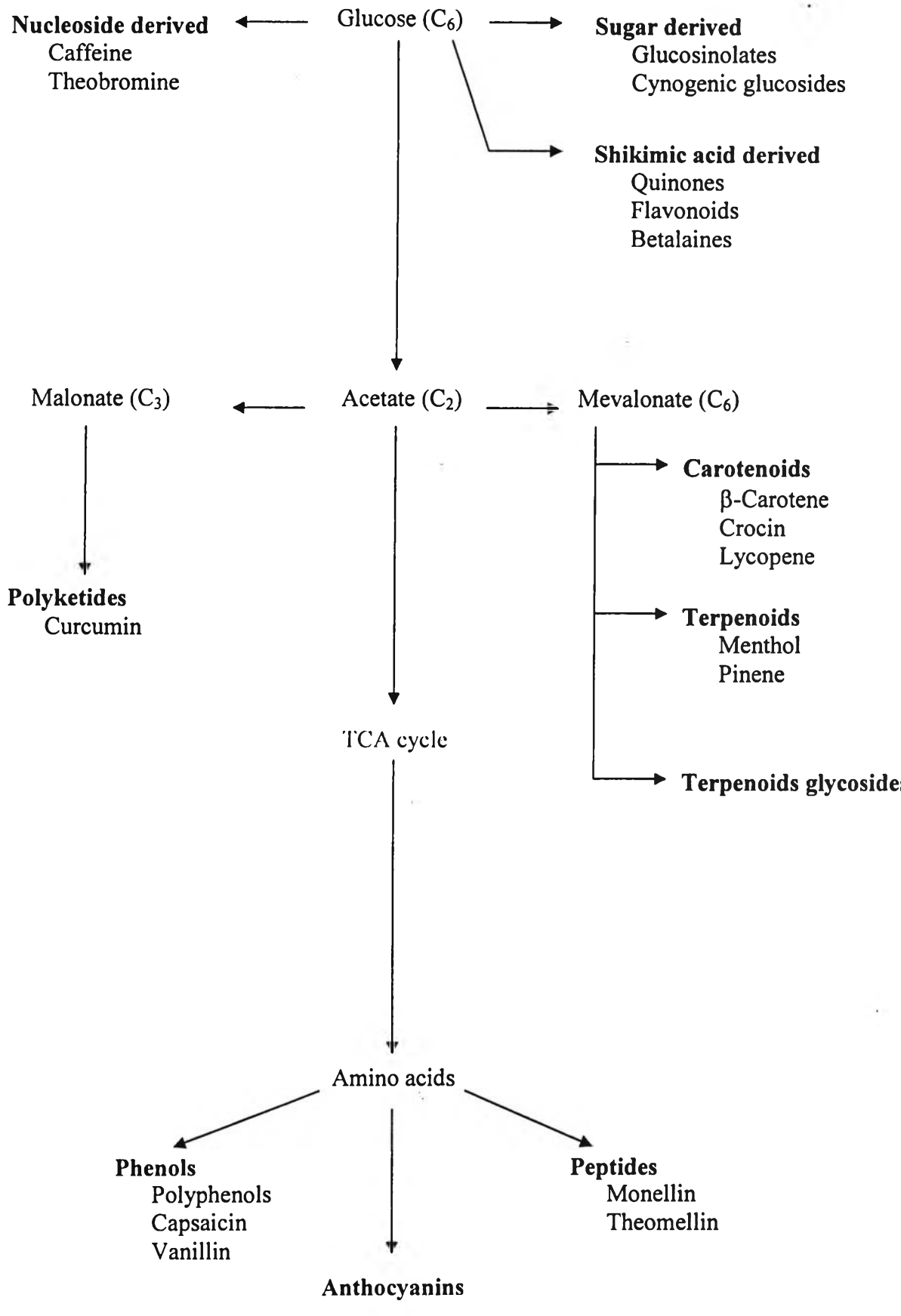


Figure 3 Schematic diagram of the biosynthetic pathway for secondary metabolites

1.12 Production of secondary metabolites by plant cell and tissue cultures

Some secondary metabolites have excellent pharmacological effects and they have been widely used in medication, so the supplies from intact plants would be limited. Plant cell and tissue cultures have been applied for solving these problems. Plant cell and tissue cultures are able to provide a source of secondary products and would make the industry less reliant on an imported source of plants and enable it to regulate its own supply of raw materials more effectively.

Plant cell and tissue methods have gained considerable importance towards the production of biologically active complex organic molecules. Certain pharmaceutically important chemicals such as shikonin, digoxin, vinblastine, berberine, rosmarinic acid, artemisinin, ginkgolides, and taxol have been successfully produced by cell cultures in large bioreactors under precise growth conditions. This situation is the outcome of basic researches over the past three decades followed by recent technological innovations.

In spite of huge effort that has been put into research for production of secondary metabolites with plant cell cultures, not many products have reached the commercial stage. The main problem is that *in vitro* cultures often only produce small quantities of the desired product. A number of basic biological and technical problems related to the characteristics of plant cells, such as the enormous sizes compared to microbial cells, growth as aggregates, slow growth rate and high sensitivity to shear stress, are responsible for the low number of industrial processes and for the decrease in research activities regarding secondary metabolite production with cultured plant cells (Dörnenburg and Knorr, 1995).

Cell suspension cultures and callus cultures, derived from plant species that normally accumulated a wide range of secondary products, showed that the concentration of the most important secondary products were all uniformly low. In addition, profiles of secondary products in tissue cultures were often unlike those in mature plant. Subsequently, research was directed towards increasing yields of secondary products in callus and cell suspensions. The approach was the same as that used to boost yields in large scale microbial systems. It was based on a selection for high yielding cells and modifications for large-scale culture of plant cells.

Recently, the secondary metabolite production by plant cell an tissue cultures has been subjected to extended research. The economic production of valuable compounds from plant cell and tissue has been a major purpose in studying the

biosynthesis of desired secondary metabolites. The selected examples with higher yields than parent plants are list in Table 3

Table 3 Secondary metabolites produced in high levels by plant cell and tissue cultures (Dicosmo and Misawa, 1995)

Compounds	**Plant species**	**Yield (% dry weight)**	
		Cell cultures	**Whole plants**
Shikonin	*Lithospermum erythrorhizon*	20 (s)	1.5
Ginsenoside	*Panax ginseng*	27 (c)	4.5
Anthraquinone	*Morinda citrifolia*	18 (s)	2.2
Ajmalicine	*Catharanthus roseus*	1.0 (s)	0.3
Rosmarinic acid	*Coleus blumeii*	15 (s)	3
Ubiquinone-10	*Nicotiana tabacum*	0.036 (s)	0.0003
Diosgenin	*Dioscorea deltoidea*	2 (s)	2
Benzylisoquinoline	*Coptis japonica*	11 (s)	5-10
Berberine	*Thalictrum minor*	10 (s)	0.01
Berberine	*Coptis japonica*	10 (s)	2-4
Anthraquinones	*Galium verum*	5.4 (s)	1.2
Anthraquinones	*Galium aparine*	3.8 (s)	0.2
Nicotine	*Nicotiana tabacum*	3.4 (c)	2.0
Bisoclaurine	*Stephania cepharantha*	2.3 (s)	0.8
Tripdiolide	*Tripterygium wilfordii*	0.05 (s)	0.001

(c) = callus cultures

(s) = cell suspension cultures

1.13 Effect of culture conditions on secondary product synthesis

In most batch cell suspension cultures, secondary product accumulation tends to increase at the end of the period of rapid cell division of the growth cycle. Even in cultures of species where accumulation of secondary products is growth related, inhibition of the growth of the culture leads to an increase in secondary production accumulation.

There appears to be an inverse correlation between growth and secondary product formation in both patterns of synthesis. It is necessary for cells to grow slowly for maximum secondary production synthesis to occur. Under conditions of limited growth, the concentration of secondary products show an increase but total amount may be small if the biomass is limited.

In order to achieve maximum secondary product formation in cell culture, it would be necessary to accumulate a large initial biomass followed by a period in which growth is inhibited. This would require a two-stage culture process in which the initial medium is designed to stimulate growth and rapid accumulation of biomass, followed by a production medium which is nutrient-limited but that contains a high concentration of sucrose.

1.14 Strategies for improving the production of secondary metabolites in cell cultures

In order to improve yield of secondary metabolite produced by plant cell culture, some strategies have been applied to plant cell.

1.14.1 Selection of high-yielding cell lines

The selection of high-yielding cell lines has been a major factor in countering low productivity. One of the most important stages in the production of secondary metabolites is selection of productive cells. Selection should be coupled with optimisation of culture conditions. If selection was to be discontinued, the culture would become more and more low-yielding because fewer cells would continue to be productive, most would revert to an unspecialised state and carry out only primary metabolism. Thus it is important to continue screening for productive cells throughout the operation of the process (Hunter, 1993).

Yield per cell are determined by a complex interaction of factors. These are the cell genotype, cell age, position of cell in an aggregate, the level of differentiation of the cells in tissue and composition of the media. In cell culture the genotype of any one cell may differ from that of the parent cells as a result of somaclonal variation. Since somaclonal variation can affect the ability of cells to synthesised secondary products, a culture may contain cells with a wide range of biosynthetic capacity. This variation provides the basis for selection for increased yields by a number of methods. In case of plant cell cultures can produces coloured secondary products, the direct visual is the best method for screening. Most secondary products are colourless,

although some can be visualised under ultraviolet whilst others fluoresce under certain other lighting conditions. Other screening methods have relied on radio-immunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), chemical or biological assays.

An elegant way of screening in some cases is to use precursor feeding. In some plant cell culture systems precursors of desired product are more toxic to the cells than the product. Hence those cells that are most productive will survive by converting the toxic precursor to the less toxic product, whereas non-productive cells will be killed by the toxic precursor.

In many cases, however, detecting the product within the cell, without destroying the culture, has proved to be so difficult that this stage has had to be omitted.

The problem with all such selected lines is stability and reversion (such as is common for high-yielding and selected microbial strains) may occur. It is well known that changes in genetic characteristics of cells occur within a culture so that callus selected for specific biochemical properties may need reselection after a period of times. For plant cells this rate of reversion far exceeds the rate of spontaneous mutation and this suggests that many of the selected lines may be epigenetic variant rather than true genetically different subspecies. However clonal screening is not valueless as over a number of cycles of repeated screening it should be possible to isolate either mutants or variants in which the epigenetic state is stabilised. Indeed, the commercial exploitation of plant cell cultures demonstrates this possibility (Banthorpe, 1994).

Selected examples of plant species, secondary metabolites, and selection method studied in plant cell cultures are presented in Table 4.

Table 4 Selected examples of plant species, secondary metabolites, and selection methods studied in plant cell cultures

Plant species	Secondary metabolites	Selection methods	References
Nicotiana tabacum	Nicotine	Single cell cloning	Ogino *et al.*, 1978
Catharanthus roseus	Anthocyanins	Optical microspectrophotometry	Hall and Yeoman, 1986
100 *Papaver* spp.	(S)-and (R)-Reticuline, salutaridine, thebaine, codeine, and morphine	Radioimmunoasay (RIA)	Wieczorek *et al.*, 1986
Daucus carota	Anthocyanins	Clonal screening	Dougall and Vogelien, 1990
Artemisia annua	Artemisinin	Clonal screening	Kumar, 1996

1.14.2 Cell immobilisation

The immobilisation of plant cell cultures for production of secondary metabolites has been investigated extensively because of the obvious advantage for use in biotechnological process. Callus and cell suspension cultures are usually run as batch cultures but immobilized cell cultures have been developed that can be continuously operated over extended periods.

The technique of cell immobilisation has been explored both in its own right in attempts at enhancing product yields and as a procedure that could possibly be exploited commercially (Banthorpe, 1994). It is commonly found that the less organised and differentiated the culture, the lower the yield of secondary metabolites. One possible way of overcoming this problem would be to increase the degree of cell organisation in bioreactor system. The aim is to increase cell-to-cell communication by immobilising the cells in a matrix (Hunter, 1993).

The principle of cell immobilisation is cells which are enclosed in an inert material, so that they are still in contact with each other and maintained in a medium that only allows a limited growth. Usually the medium has reduced phosphate, nitrate and growth regulator supply, and a large increase in the sucrose level. This medium is circulated around the immobilised cells, so that secondary products released into the medium can be removed, thus reducing any feedback inhibition. Since the immobilised cells are growing very slowly, they can be maintained as an active production until for up to 30 weeks compared with 3-4 weeks for a normal cell suspension culture (Collin and Edwards, 1998).

The immobilisation method may vary but all retain the principle of providing a protected environment for the cells (Collin and Edwards, 1998)

1. Nylon sheets are folded to enclose the cells then the sheets are maintained in a bioreactor with an air lift system to circulate the nutrients.
2. Porous foam blocks are included in liquid medium containing a cell suspension. Cells grow into the spaces within the blocks, these blocks are then maintained as a bed while medium is circulated around them by airlift system.
3. The cells are enclosed in a calcium alginate gel formed by adding drops of cell suspension and sodium alginate to calcium alginate solution. Spheres of calcium alginate enclosing the cells can then be maintained in a fixed-bed column with medium trickling over the beads.

All these systems aim towards increasing the degree of cell-to-cell contact whilst limiting growth and maintaining a relatively easy-to-manipulate bioreactor culture (Hunter, 1993).

A summary of the advantages of immobilised plant cell system is shown as below: (Yeoman *et al.*, 1990)

1. Process can be run continuously as long as the metabolite is released from the cells and is removed from the medium
2. Rapid changes can be made to the medium circulating around the immobilised biomass, substances can be added or removed quickly and easily
3. The biomass can be rejuvenated (in some systems) in situ by perfusing the cells at intervals with growth medium
4. The proportion of time spent growing cells is low compared to the time spent in the production phase
5. Efficient use of relatively small amounts of biomass
6. Cells can immobilised easily, either passively or actively
7. Structural and biochemical differentiation can be achieved within the inert matrix

Selected examples of plant species, secondary metabolites, and immobilization method studied in plant cell cultures are presented in Table 5

Table 5 Selected examples of plant species, secondary metabolites, and immobilisation methods studied in plant cell cultures

Plant species	Secondary metabolites	Immobilisation methods	References
Pelargonium fragrans	Monoterpene hydrocarbons	Calcium alginate beads	Brown and Charlwood, 1986
Thalictrum rugosum	Berberine	Agarose	Brodelius *et al.*, 1988
Anethum graveolen	Geraniol, nerol	Nylon mesh	Everitt and Lockwood, 1992
Papaver somniferum	Sanguinarine	Surface immobilisation bioreactor	Archambault *et al.*, 1996
Mentha piperita	Essential oil	Polyurethane foam	Ha *et al.*, 1996

1.14.3 Morphological modifications

According to the dedifferentiation of plant tissue *in vitro* to produce callus and suspension cells is usually accompanied by an apparent loss of ability to accumulate secondary compounds. The reasons for such non-accumulation may be: (Charlwood *et al.*, 1990)

1. The lack of expression in non-specialised cells of genes that control the essential steps in the biosynthetic pathway;
2. The diversion of substrate away from secondary product formation;
3. The non-operation of transport mechanisms by which potentially toxic end products may be removed from the biosynthetic site;
4. The non-availability of storage sites in which secondary metabolites would normally be sequestered;
5. The unregulated catabolism of synthesised product.

The process of secondary metabolism is mediated through several enzymatic regulations, which are primarily controlled by the genetic make up of the cells. The type and degree of differentiation plays an important role in the biosynthetic events. The *in vitro* cultures undergo various types of differentiation, which eventually lead to changes in the metabolic events.

In the transition from simple cells and small cell aggregates in a cell suspension to larger aggregates, then differentiation into roots, shoots or embryos, the more complex tissue shows an increased capacity for secondary product accumulation (Collin and Edwards, 1998).

This improvement in secondary product accumulation seems to be associated with cell-cell contact and the increased complexity of cells and cell structures that are found in differentiated tissue.

Organogenesis means formation of organ such as root, stem, leaf, and flower. Plant organ cultures are characterised by their maintenance of structure integrity, as a consequence of growth from a defined meristem, and are thereby distinguished from callus and suspension cultures. Such morphological stability is in turn associated with improved genetic and metabolic stability compared with undifferentiated culture.

Morphological modifications are classified into two classes, which are organ cultures and transformed cultures.

Organ cultures that are shoot and root culture have been shown to accumulate secondary metabolites. A callus is usually more capable of regenerating adventitious

roots than adventitious shoots (Thomas and Davey, 1975). Root formation generally takes place in a medium with a relatively high auxin and low cytokinin concentration. The initiation of root primordial often requires a higher auxin concentration than that required for the out growth of root primordial. Roots and shoots are usually formed completely independently from each other i.e. there is no direct link between them if they arise from the callus at the same time.

Adventitious shoot formation can appear in callus tissue if there is a low auxin concentration and high cytokinin concentration. BA is the most effective cytokinin for inducing the formation of adventitious shoot. In some cases adventitious roots are formed at the base of adventitious shoots (Pierik, 1993).

Thidiazuron (TDZ) (Fig. 4) is non-purine cytokinin-like compound. It has been shown to exhibit stronger effects than conventional cytokinins over a wide range species. It is effective for axillary shoot proliferation and adventitious shoot organogenesis (Huetteman and Preece, 1993). Its mode of action may be attributed to its ability to induce cytokinin accumulation and translocation of auxin within TDZ exposed tissue (Murch and Saxena, 2001).

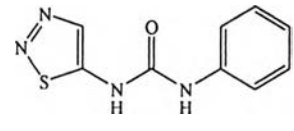


Figure 4 Chemical structure of thidiazuron (TDZ)

Transformed plant organ cultures have proved to be valuable in the study of aspects of secondary metabolism. Their advantages over conventional cell suspension cultures lie in their genetic and biochemical stability over long periods in culture and the potential for introducing novel genes to modify growth and secondary metabolism. Transformed cultures of both roots and shoots have been developed. They are derived following infection with the plant pathogens, *Agrobacterium rhizogenes* or *Agrobacterium tumefaciens* (Rhodes *et al.*, 1994). The bacteria enter the plant through wounds in the epidermis, then the bacteria plasmid, Ri or Ti, respectively, transfers a portion of its DNA, the T-DNA into the plant cell and ultimately the T-DNA becomes incorporated into the plant genome. Expression of the host plant genome is modified by expression of the bacterial genes which cause overproduction of auxins and cytokinins. This leads to the production of gall and proliferation of

roots. These roots can be excised and if place on a simple inorganic medium will grow rapidly and continuously. The secondary product level is also comparable to that of plant *in vivo* and much higher than in cell suspensions (Collin, 1998).

The advantage of transformation is that transformed cultures have no phytohormone requirement and generally show good stability in culture. Furthermore, as shown now for a number of cases, genes can be inserted into the plant allowing the pathway to be altered, potentially providing new chemical species. On the other hand, two major problems are encounter when using *Agrobacteria*, these are susceptibility and hypersensitivity of some plant cells or tissues (Robin, 1998).

Selected examples of plant species, secondary metabolites, and organogenesis had been studied in plant cell cultures are presented in Table 6

Table 6 Selected examples of plant species, secondary metabolites, and organogenesis studied in plant cell cultures

Plant species	Secondary metabolites	Organogenesis	References
Rosmarinus officinalis	α-Pinene, β-pinene	Shoot culture	Webb *et al.*, 1984
Mentha citrata	Linalool, linalyl acetate	Transformed shoot cultures	Spencer *et al.*, 1990
Rauwolfia serpentiana	Reserpine, ajmalicine, ajmaline, serpentine	Organogenesis	Bejamin *et al.*, 1993
Artemisia absinthium	Neryl isovalerate, neryl butyrate, nerol, linalyl-3-methylbutanoate	Transformed root culture	Kenedy *et al.*, 1993 Nin *et al.*, 1997
Hyoscyamus muticus	Hyoscyamine	Transformed root cultures	Oksman-Caldentey *et al.*, 1994
Valeria officinalis var. *sambucifolia*	Bornyl acetate, valerenal	Root culture	Fraenicher *et al.*, 1995
Pimpinella anisum	trans-Epoxypseudoisoeugenyl 2-methylbutyrate, geijerene, pregeijerene, zingigerine, β-bisabolene	Transformed root cultures	Santos *et al.*, 1998
Lavandula dentata	1,8-cineole, fenchol, borneol, camphor	Shoot cultures	Sudria *et al.*, 1999

1.14.4 Precursor feeding and biotransformation

Biotransformation is a process through which the functional groups of organic compounds are modified by living cells, and is a useful tool in the study of applications of plant cell culture. In the last 30 years, much research was related to this topic and the potential of plant cell cultures for biotransformation process were realised. The most advanced example of biotransformation with an industrial application, being the hydroxylation of β-methyldigoxin to β-methyldigitoxin, was developed by Reinhard and Alfermann (Reinhard and Alfermann, 1980). The enormaous biochemical potential of plant cells, which perform specific biotransformation on particular natural or synthetic substrates to produce more valuable substances, leads to many possible applications.

Cell suspension cultures can be used additionally for biotransformation of added substrates, to search for new compounds not yet found in nature, and for the isolation of enzymes, which may be attractive for chemical synthesis of natural products. Furthermore, plant cell culture techniques are important for production of cells, as well as plants producing recombinant proteins.

However, problems are still retained, such as slow rate of growth, difficulty of maintaining culture sterility, and the lower yield of products. In some cases, two-stage systems are used, which is where firstly a growth medium is used to produce biomass quickly, followed by a production medium to stimulate formation of secondary metabolites.

Recently, biotransformation combined with immobilisation and precursor feeding is widely used for commercial purpose.

Since de novo biosynthesis has not been successful, in most cases, biotransformation of added precursors has been studied extensively to produce valuable compounds, partly with the goal of finding possible blocks in biosynthetic products. Suspension cultures are able to achieve certain transformations. In many cases, a precursor is submitted to multi-step bioconversions, resulting in production of unknown products. The range of compounds which can be used for biotransformation by plant cells in extensive, for instance, plant cell cultures have the potential for the bioconversion of phenylpropanoids, mevalonates, and alkaloids. The reaction types include reduction, oxidation, hydroxylation, acetylation, epoxidation, glycosylation, (de)esterification, (de)methylation, and isomerisation.

In the study of these precursors, mevalonates including terpenoids, steroids, and saponins may be the most common substrates for biotransformation using plant cell cultures. Precursors of terpenoids and steroids are derived from mevalonic acid. Among these, biotransformation of monoterpenoids has attracted most attention in recent years because they are volatile substances responsible for much of the characteristic aromas of plants. The bioconversion of these compounds may take quite a short time, ranging from 15-25 minutes for the acyclic monoterpenoids geranial, neral, and citronellal, and up to 75 minutes for the acyclic monoterpenoid perilladehyde (Scragg *et al.*, 1991 a)

There are various conditions required for successful and viable process (Scragg, 1991 b)

1. The substrate of a biotransformation reaction must be easily assimilated by the cell and reach the appropriate cellular compartment or organelle without significant degradation.
2. The substance must not be toxic to the cell culture.
3. The rate of product formation must significantly exceed the rate of its further metabolism.

Table 7 shows selected examples of plant species, secondary metabolites, precursors and biotransformation products had been studied in plant cell cultures.

Table 7 Selected examples of plant species, secondary metabolites, precursors and biotransformation products studied in plant cell cultures

Plant species	Precursors	Biotansformation products	References
Vitis vinifera	Citral	Its correspondence alcohol; nerol and geraniol	Ambid *et al.*, 1982
Nicotiana tabacum	3-Carene	3,4-epoxycarene, 3-caren-5-one, 3,4-caranediol, 3,6,6-rimethylcycloheptadien-1-one, 8-hydroxy-m-cymene	Hirata *et al.*, 1994
Catharanthus roseus	Geraniol, nerol, (+)-carvone, (-)-carvone	5β-Hydroxyneo-dihydroxycarveol	Hamada *et al.*, 1997
Eucalyptus perriniana	(+)-Camphor	(1S, 4R, 6S)-6-Hydroxybornan-2-one 6-O-b- D-gluco-pyranoside	Orihara *et al.*, 1994
Mentha piperita	(+)-Isopiperitenone (-)-(4R)-Carvone (+)-(4S)-Carvone	(4S,6R)-6-Hydroxy- and (4S,8R)-8,9-epoxyisopiperitenone (1R,2S,4R)-Neodihydrocarveol, (1R,2R,4R)-dihydrocarveol (1S,2R,4S)- Neodihydrocarveol, (1S,4S)-dihydrocarvone	Kim *et al.*, 2002
Picea abies	(1R)-(+)-α-Pinene	(1R)-trans-Verbenol, (1R)-cis-verbenol, (1R)-(+)-verbenone, (1S)-(-)-pinocarveol, (1S)-(+)-myrtenol, (4R)-(+)-α-terpineol	Lindmark-Henriksson *et al.*, 2003

1.14.5 Elicitation

Elicitation is defined as a method whereby cells are cultured through a modified environment with the objective of perturbing metabolism and thus increasing the production of secondary metabolites (Yeoman and Yeoman, 1996).

Elicitors may form inside or outside of plant cells, and are distinguished as either endogenous or exogenous inducer. Also, according to their origin, they are classified as biotic and abiotic elicitor (Dörnenburg and Knorr, 1995). Biotic elicitors mainly consist of chitosan (Chang *et al.*, 1998), glucans and glycoproteins, and abiotic elicitors vary from physical and chemical stresses including heavy metal ions, organic solvents, detergents, pesticides, electroporation, ultrasonic (De, 2001) or ultra-high pressure treatment, and pH change. In recent year microbial elicitors (Kombrink and Hahlbrock, 1986) have received increased attention, because of their ability to increase the yield of a target substance, and also advance studies of enzymology of secondary metabolism, and studies on plant defense mechanisms. Elicitors are signals triggering the formation of secondary metabolites. Secondary pathways are activated in response to stress. The use of biotic or abiotic elicitors to stimulate product formation has become an important process strategy and has been very useful in reducing the process time to attain high product concentration and increased volumetric productivity (Dörnenburg and Knorr, 1996). It is of great importance to establish an optimum amount of elicitor to added to the culture, and the growth stage of cells in another important factor for optimal effect of elicitor treatments.

The selected examples of plant species, secondary metabolites, and elicitors studied in plant cell cultures have been reviewed in Table 8.

Table 8 Selected examples of plant species, secondary metabolites, and elicitors studied in plant cell cultures

Plant species	Secondary metabolites	Elicitors	References
Eschscholtzia californica	Chelerythrine, macarpine	Chitosan	Brodelius *et al.*, 1989
Ruta graveolens	Acridone epoxide, furoquinolines, furanocoumarine	Yeast extract	Koprek *et al.*, 1992
Gossypium hirsutum	(+)-δ-Cadinene	Bacterial elicitor	Davis and Essenberg, 1995
Mentha piperita	Menthol	Chitosan	Chang *et al.*, 1998
Artemisia annua	Artemisinin	Fungal elicitor	Liu *et al.*, 1999
Petroselinum crispum	Butylidenephthalides	Pep25	Hagemeier *et al.*, 1999
Agastache rugosa	Methyl chavichol	Methyl jasmonate	Shin *et al.*, 2001

Yeast extract = *Rhodotorula rubra*

Bacterial elicitor = *Xanthomonas campestris* pv. *malvacearum*

Fungal elicitor = *Penicillium chysogenum*

Pep 25 = *Phytophthora sojae* 25-amino acid oligopeptide

1.14.6 Permeabilisation

The excretion behaviour of plant cell cultures varies from one species to another, and even in one species, from one cell line to another. When the cells excrete their products, the product concentrations in the reaction mixture are very low, which is an extra cost factor for their downstream processing. Various chemical treatments can be used to permeabilised plant cell and produce release of intracellular constituents, but in a reversible manner, so that cell can be maintained (Felix, 1982).

Permeabilisation depends on the formation of pores in one or more of the membrane systems of the plant cell, enabling the passage of various moleculres into and out of the cell. Various methods related to permeabilisation, include treatment solution of high ionic strength, permeabilisation with dimethylsulfoxide (DMSO) and polyoxyethylenesorbitane monolaurate (Tween-20).

However, the concentration of DMSO required to stimulate release of secondary metabolites is generally toxic to plant cells. Thus, although cell products can be recovered with the use of DMSO, the cells are destroyed by the process and cannot be reused. This limits the value of using this organic solvent.

There is an advantage that cells treated intermittently can be permeabilised repeatedly, thus permitting the maximum use of the cells biosynthetic capacity.

The selected examples of plant species, secondary metabolites, and permeabilisation studied in plant cell cultures have been showed in Table 9.

1.14.7 Two-phase system

A two-phase system, which is an artificial site, added to medium so that equilibrium is destroyed. More secondary metabolites are released from vacuolar compartments of the cell, and traces of secondary metabolites from the culture medium are accumulated (Banthorpe, 1994).

In two-phase system, one phase is an aqueous phase or aqueous medium, and the other phase is non-aqueous phase or water immiscible organic solvent such as n-hexadecane or Miglyol® (triglyceride). Agents and adsorbents are often used in two-phase system also, such as a lipophillic carrier ion exchanger, or neutral resin. Some neutral resin such as XAD, not only adsorb the excreted products from the medium, but enhance the total production as well, and recovery and purification are generally simplified, thus reducing production cost. The second phase must be tailored to each substance and be non-toxic to the cells (Banthorpe, 1994).

Two-phase culture systems for plant cells has been used with success for the continuous extraction and accumulation of lipophillic constituents including some monoterpenes out of the culture medium (Cormier and Ambid, 1987).

The advantage of this method is secondary metabolite may be protected from degradation in the culture medium as a result of excreted catabolic enzymes and acids, and desired plant products can be moved selectively from the culture system (Payne *et al.*, 1988).

Table 10 shows selected examples of plant species, secondary metabolites, and two-phase system had been studied in plant cell cultures.

Table 9 Selected examples of plant species, secondary metabolites, and permeabilisation studied in plant cell cultures

Plant species	Secondary metabolites	Permeabilisation	References
Thalictrum rugosum *Chenopodium rubrum*	Berberine and betacyanin	Electric pulse (electroporation)	Brodelius *et al.*, 1988
Datura innoxia	Hyoscyamine and scopolamine	Tween 20	Boitel-Conti *et al.*, 1995

Table 10 Selected examples of plant species, secondary metabolites, and two-phase system studied in plant cell cultures

Plant species	Secondary metabolites	Two-phase systems	References
Valeriana wallichii	Valepotriates	Aqueous nutrient medium / RP-8	Becker and Herold, 1983
Vitis vinifera	Geraniol and its biotransformation products	Aqueous nutrient medium / Miglyol® 812	Courmier and Ambid, 1987
Nicotiana tabacum	Data not shown	Dextran / polyethylene glycol	Hooker and Lee, 1990
Humulus lupulus	Data not shown	Aqueous nutrient medium / Amberlite resin (XAD-1180)	Langezaal and Scheffer, 1992
Lavandula angustifolia	Monoterpenoids	Aqueous nutrient medium / Miglyol® 812 and n-hexadecane	Banthorpe *et al.*, 1995

1.14.8 Used of adsorbent

Secondary metabolites especially, essential oils, in intact plants are accumulated in specialised secretory organs, and these can not be formed in undifferentiated cells such as callus or suspension cultures, so volatiles constituents will be released from cells into culture media. Some adsorbents were applied for collecting these volatiles such as Porapak Q (ethyl vinyl benzene-divinyl benzene copolymer), Tenax GC (2,6-diphenyl-p-phenylene oxide polymer), charcoal, and methanol in dry ice (Faldt, 2000). The sampling result is related to the properties of sorbent materials and depends on the pore size and chemical properties of the polymer. Compare to Tenax GC, Porapak Q can adsorb larger amounts of analyte, or different proportions (Lockwood, 2001).

The adsorption of the compounds to polymers is based on physical and chemical parameters of compounds and adsorbents. *Purge and Trap* is the method referred to the process of collecting volatile oil in air stream by trapping them on a sorbent placed in the outlet stream, then desorbing them by the solvent.

Zhu (Zhu, 2000) has used Porapak Q in plant tissue cultures by packing it into the glass tube and collecting volatile constituents released from callus and cell suspension cultures. After using this technique, the collected volatiles have been increased.

1.15 Essential oils

Essential oils are the odorous principles found in various plant parts. Because they evaporate when exposed to the air at ordinary temperatures, they are called volatile oils, ethereal oil, or essential oils (Tyler *et al.*, 1988).

Depending on the plant family, essential oils may occur in specialise secretory structures such as glandular hair (Labiatae), modified parenchyma cells (Piperaceae), oil-tube call vitae (Umbelliferae), or lysigenous or schizogenous passage (Pinaceae and Rutaceae). They may be formed directly by protoplasm, by decomposition of the resinogenous layer of the cell wall, or by the hydrolysis of certain glycosides (Tyler *et al.*, 1988).

Essential oils may act as repellents to insects, thus preventing the destruction of the flowers and leaves; or they may serve as insect attractants, thus aiding in cross-fertilisation of flowers (Tyler *et al.*, 1988).

Chemical constituents of essential oils may be divided into 2 broad classes, based on their biosynthesis origin (Tyler *et al.*, 1988):

1. Terpene derivatives formed via the acetate-mevalonic acid pathway
2. Aromatic compounds via the shikimic acid-phenylpropanoid pathway.

Many essential oils consist largely of terpenes, and the most often found in essential oils are monoterpenes.

1.16 Biosythesis of essential oil constituents

The biosynthetic building blocks for terpenes are isoprene units. The biosynthetically active isoprene units are called isopenthenylpyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), compounds arise from acetate via mevalonic acid (Fig. 5). Geranyl pyrophosphate is C-10 precursor of terpenes and is belived to play a key role in the formation of monoterpenes. It is formed by the condensation of one unit each of IPP and DMAPP.

As seen in Fig. 5, geranyl pyrophosphate is believed to be the direct precursor to acyclic monoterpenes. However, it must be isomerised to neryl pyrophosphate before the cyclic monoterpenes can be formed because the *trans* isomer does not have the correct stereochemistry for cyclisation. Another possibility is the formation of neryl pyrophosphate from IPP and DMAPP independent of geranyl pyrophosphate step. The intermediates in the formation of cyclic terpenes are shown as carbonium ions; however the true species are probably pyrophosphate esters or enzymes-bound intermediate (Tyler *et al.*, 1988).

The principal precursors for phenylpropanoid compounds, which are found in essential oils, are cinnamic acid and *p*-hydroxycinnamic acid, also known as *p*-coumaric acid. In plants, these compounds arise from the aromatic amino acid phenylalanine and tyrosine, respectively, which in turn are synthesised via the shikimic acid pathway (Fig. 6). This biosynthetic pathway has been elucidated in microorganism by using auxotropic mutants of *Escherichia coli* and *Enterobacter aerogenes* that require the aromatic amino acids for growth. In the biosynthesis, two glucose metabolites, erythrose 4-phosphate and phosphoenolpyruvate, react to yield a phosphorelated 7-carbon keto sugar, 3,deoxy-D-arabinoheptulosonic acid-7-phosphate (DAHP), this compound cyclises to 5-dehydroquinic acid, which is then converted to shikimic acid. Shikimic acid, through a series of phosphorylated intermediates, yields chorismic acid, which is an important branch-point intermediate. One branch leads to

anthranillic acid and then to tryptophan. The other leads to prephenic acid, the last non-aromatic compound in the sequence. Prephenic acid can be aromatised in two ways. The first proceeds by dehydration and simultaneous decarboxylation to yield phenylpyruvic acid, the direct precursor pf phenylalanine. The second occurs by dehydrogenation and decarboxylation to yield p-hydroxyohenylpyruvic acid, the precursor of tyrosine. The phenylpropanoid precursor, cinnamic acid is formed by the direct enzymatic deamination of phenylalanine, and p-coumaric acid can originate in an analogous way from tyrosine or by hydroxylation of cinnamic acid at the *para* position (Tyler *et al.*, 1988).

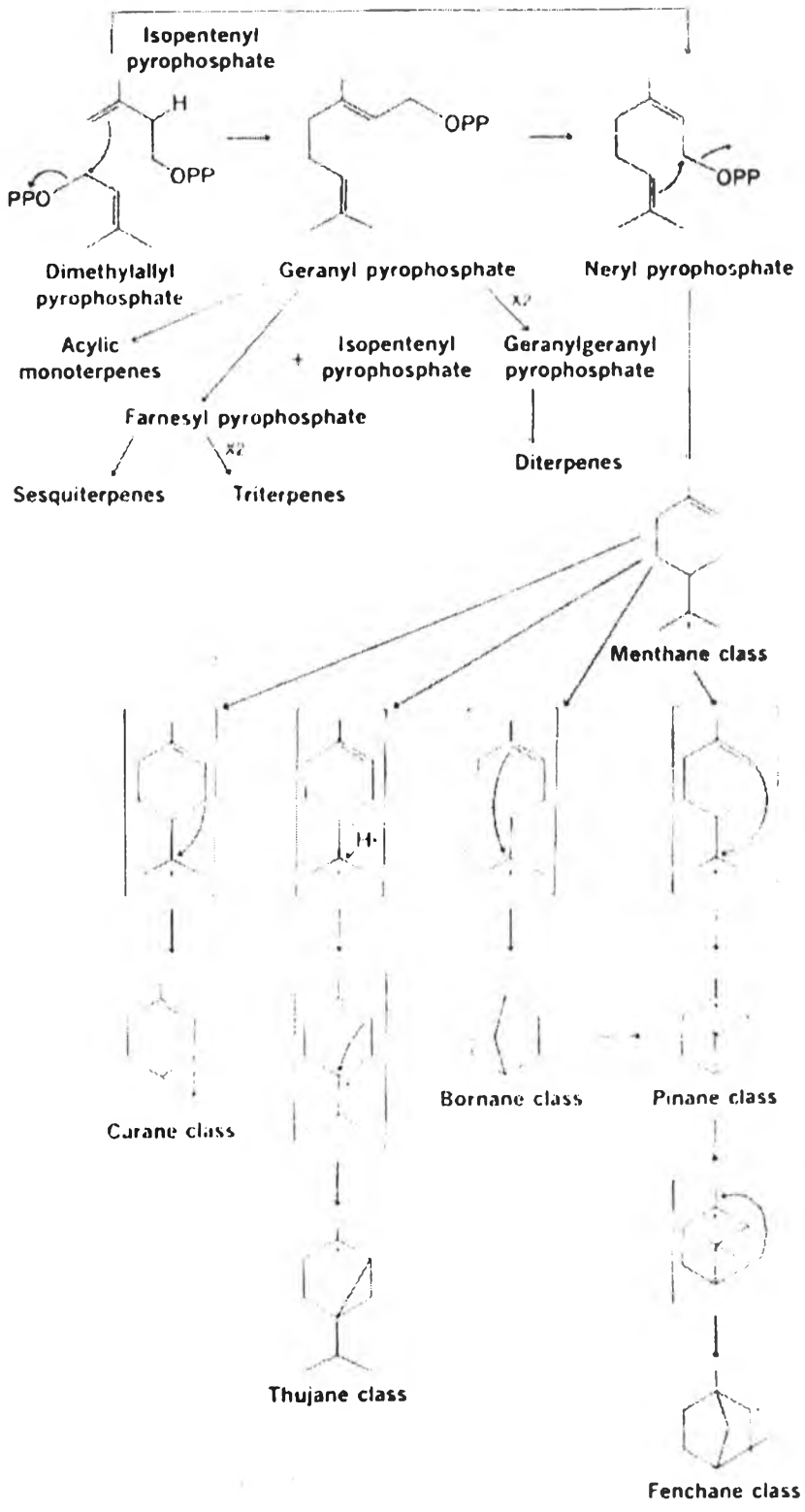


Figure 5 Biosynthesis pathway of terpenes via acetate-mevalonic acid (Tyler *et al.*, 1988)

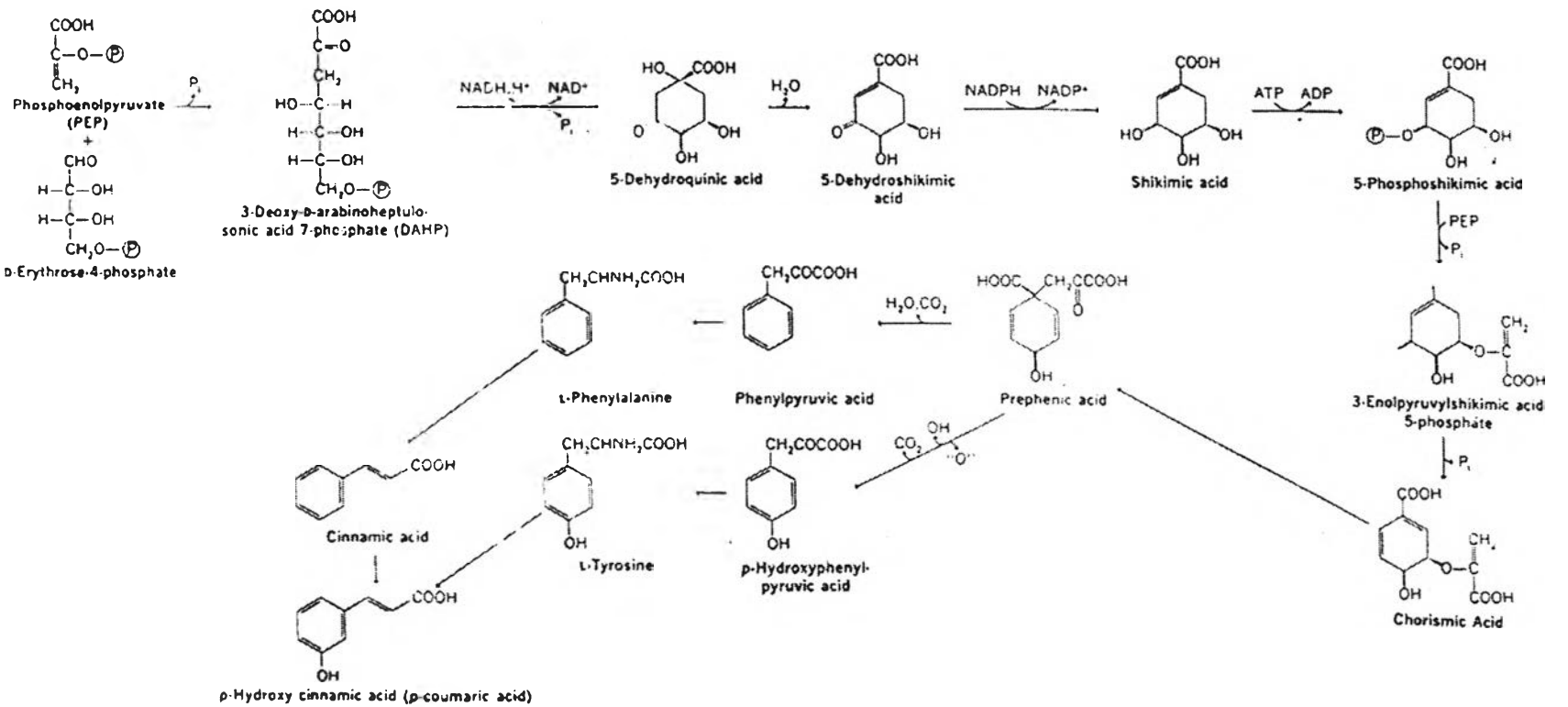


Figure 6 Biosynthesis pathway of phenylpropanoid compounds (Tyler *et al.*, 1988)

1.17 Medicinal and commercial uses

Practically all essential oils consist of chemical mixtures that are often quite complex, which are important groups of natural products with industrial interest. They have been used in perfumery and cosmetics as aroma products; in food and beverage as flavours, and medicine (Tyler *et al.*, 1988).

Many crude drugs are used medicinally because of their volatile oil content, however, in numerous cases the volatile oils separated from the drugs are used as drugs themselves.

For therapeutic purpose, they are administered as inhalations (e.g. eucalypus oil), oral (peppermint oil), as gargles and mouthwashes (e.g. thymol) and transdermally (many essential oils including those of lavender, rosemary, and bergamot are employed in the practice of aromatherapy) (Evan, 1996).

The oil with a high phenol content, e.g. clove and thyme have antiseptic properties, where as other are used as carminatives. Oils showing antispasmodic activity, and much used in popular medicine, are those of *Melissa offocinalis*, *Rosmarinus officinalis*, *Mentha piperita*, *Matricaria chamomile*, *Foeniculum vulgarae*, *Carum carvi* and *Citrus aurantium* (Evan, 1996).

The fabrication of perfumes is multimillion-dollar. Perfumery materials such as volatile oils are used directly not only for perfumes and cosmetic but also are essential for the manufacture of soaps, toiletries, and deodorizers and for masking or proving odour to house hold cleaner, polishes and insecticides (Tyler, 1988).

1.18 Production of essential oil from plant cell cultures

Plant cell cultures, have been used for the procurement of natural raw material. Production of essential oils as secondary metabolites in plant cell cultures has been reviewed (Mulder-Krieger *et al.*, 1988, Banthorpe, 1990, Shin, 1995, Sansongsak and Lockwood, 2004). It has proved to be difficult and most cultures have been unable to produce adequate yields of flavour substances, possibly because of the fact that most essential oils in intact plants are accumulated in specialised secretory organs, and these can not be formed in callus and suspension cultures. More often, dedifferentiation of plant cells is accompanied by a partial of total loss of ability to accumulate essential oils. The poor accumulation by unorganised cells could be caused by many factors, such as, the diversion of carbon flux from the secondary

pathways, the non-availability of transport mechanisms or storage sites for the end product, or the unregulated breakdown of newly synthesised compounds.

It is known that monoterpenes are cytotoxic to plant cells, causing a decrease in cell membrane permeability and inhibiting respiration and photosynthesis (Brown *et al.*, 1987). Also, a significant correlation between the accumulation of essential oil and density of storage sites has been noted.

In intact plants, toxic compounds are sequestered and storaged at specialised sites, including glandular hair, resin canals, and schizogenous glands, which are not found in undifferentiated cells.

If there is any correlation between accumulation of essential oils in undifferentiated cells and toxicity, it should be possible to enhance the productivity by establishment of artificial storage sites for them.

The synthesis of essential oils in plant cell cultures will possibly be stimulated by:

1. Induction of morphological differentiation in cell culture
2. Creating of artificial accumulation sites for essential oils.

According to this point of view, many biotechnological methods have been utilised to enhance the yield of essential oils, these are:

1. Selection of high-yielding cell line
2. Cell immobilisation
3. Organogenesis
4. Precursor feeding and biotransformation
5. Elicitation
6. Permeabilisation
7. Two-phase system
8. Used of adsorbent

Selected examples of essential oil production in plant cell cultures are presented in Table 11

Table 11 Selected examples of essential oil production in plant cell cultures

Plant species	Detection compound	Biotechnological methods	Reference
Achillea millefolium	Epi-cubevol, neryl isovalerate	Organogenesis (hairy root cultures)	Lourenco *et al.*, 1999
Agastache rugosa	Methylchavichol	Elicitation with methyl jasmonate	Shin *et al.*, 2001
Caragana chamlagu	Mayurone and two new compounds (3β-hydroxy-4-thujopsene and 3β-epoxythujpsa-5β-ol	Precursor (thujopsene) feeding and biotransformation	Sakamaki *et al.*, 2001
Citrus sinensis	Essential oils	Organogenesis	Niedz *et al.*, 1997
Mentha citra, Mentha piperata	Menthone, menthofuran, menthyl acetate, menthol	Organogenesis (*Agrobacterium* transformed shoot cultures)	Spencer *et al.*, 1993
Mentha piperita	Menthol	Elicitation with chitosan	Chang *et al.*, 1998
Lavendula angustifolia	β-Pinene, β-ocimene	Organogenesis (shoot regeneration)	Webb *et al.*, 1984
Origanum vulgare spp. *virens*	Essential oils	-	Alves-Pereira *et al.*, 1998
Perilla frutescens	Perilladehyde	Genetic control of enzymatic formation	Nishizawa *et al.*, 1992

Table 11 Selected examples of essential oil production in plant cell cultures (Cont.)

Plant species	Detection compound	Biotechnological methods	Reference
Pimpinella anisum	trans-Epoxypseudoisoeugenol-2-methylbutyrate, giejerene, zingiberene, β-bisabolene	Organogenesis (hairy root cultures)	Santos *et al.*, 1998
Pogostemon patchouli	Norpatchoulenol	*in vitro* Propagation	Maheshwari *et al.*, 1993
Rosa damascena	2-Phenylethanol	Organogenesis (shoot regeneration)	Banthorpe *et al.*, 1988
Rosmarinus officinalis	β-Pinene, α-pinene	Organogenesis (shoot regeneration)	Webb *et al.*, 1984
Thymus vulgaris	Thymol	-	Yamaura *et al.*, 1992
Vitis vinifera	Geraniol	Two-phase system	Cormier *et al.*, 1987