CHAPTER I
INTRODUCTION

1.1 Plant cell and tissue culture

For along time, plants have been used by man in one form or the others namely, medicines, flavours, fragrances and various other industrial and pharmaceutical products. 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 (Srivastava, 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. The *in vitro* culture of plant cells and tissues is the one of growth areas of biotechnology because of its potential to generate improved crops and ornamental plants (Srivastava, 1994).

Plant cell and tissue culture is considered a viable alternative for production, if the product formation rate, yield, and final concentration are increased significantly. Major advantages expected from cell culture systems over the conventional cultivation of whole plants are as follow: (Tabata, 1977)

1. Useful compounds could be produced under controlled environmental conditions independent of climatic changes or soil conditions.
2. Cultured cells would be free of microbes and insects.
3. The cell of any plants, tropical or alpine, could easily be multiplied to yield their specific metabolites.
4. Automate control of cell growth and rational regulation of metabolic processes would contribute to the reduction of labor costs and the improvement of productivity.

1.2 Definition

Plant cell and tissue culture are the process whereby small pieces of the living tissue (explants) are isolated from organism and grown aseptically for indefinite periods on a 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 or 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, 1991).

1.3 Callus culture

Callus culture is obtained from any part of plant, both from plant organs and from specific tissues or cells (Collin and Edwards, 1998).

The initiation of callus is from seedling, which was germinated from surface sterilized seeds, or from explants. They are transferred to semi-solid media by aseptically techniques. To maintain callus cultures is carried out by keeping them at 25°C with light and routine subculture to new media every 4-8 weeks depending on the individual cell growth.

1.4 Cell suspension culture

Suspension culture is obtained from callus culture. It can be grown in bulk, show a faster growth rate than callus culture.

The initiation of suspension culture is made by transferring callus into a liquid media with the same ingredients as that used in callus culture and agitating the cell mass breaks up to give isolated cells, small clusters of cells and much larger aggregates. To maintain suspension culture 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.

1.5 Culture medium

Culture medium is an important factor for growth and maintenance of callus and suspension cultures. The first decision to be made when initiating a plant callus culture is the composition of the culture medium. The most commonly used media are Murashige and Skoog (MS), Gamgorg B5 (B5) and Schenk and Hildebrandt (SH) (Dixon, 1996).

1.5.1 Media compositions

Components of typical plant cell and tissue culture media include (Dey and Harborne, 1997):

1. Inorganic macro nutrients (e.g. N, P, K, Ca, Mg, and Fe)
2. Inorganic micro nutrients (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. auxin and cytokinin)
7. Optional organic compounds (e.g. casein hydrolysate, yeast extract)
8. A gelling agent (0.5-1.0% w/v good quality, bacteriological grade agar) if the medium is to be semi-solid.

A variety of medium components have shown to affect product yield. Recently, researchers improved the cell growth and secondary metabolites yield by varying of media components and investigated the effect of ratio of carbon and nitrogen, sucrose and ammonia, and phosphate. It was found that nitrogen is essential for protein and nucleic synthesis, reducing or removing nitrogen will reduce the cell growth and bring into a premature stationary phase in which product accumulation. Lacking of phosphate, more than any other nutrients, stimulates secondary metabolite biosynthesis (Mantell and Smith, 1983). In general, the rising of the levels of sucrose lead to increase the secondary metabolite yield of cultures.

1.5.2 Plant growth regulators

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

For plant cells to develop into a callus, it is essential that the nutrient medium contain plant growth regulator, or plant hormones, e.g. an auxin, a cytokinin and a gibberellin. The absolute amounts of these hormones are required vary different tissue explants from different parts of the same plant and for the same explant from different genera of plants (Primrose, 1987). The type and concentration of plant regulators used will vary according to the cell culture purpose.

Most plant cells for division and root initiation requires an auxin. At high concentration auxin can suppress morphogenesis. Synthetic auxins such as 2,4-dichlorophenoxyacetic acid (2,4-D) and 1-naphthaleneacetic acid (NAA) can replace the naturally occurring auxin indole-3-acetic acid (IAA), because of IAA is readily oxidised by plant cells (Dey and Brownleader, 1997). Auxins are thermostable. However, low pH, light, oxygen, and peroxidase destroy IAA. NAA and 2,4-D are more stable forms of auxin.

A Cytokinin promotes cell division, shoot proliferation, and shoot morphogenesis. 6-Benzylaminopurine (BAP) and 6-furfurylaminopurine (kinetin) are the most commonly used cytokinins (Dey and Brownleader, 1997). Cytokinins (kinetin and zeatin) are thermostable; no breakdown products were detected after 1 hr. at 120 °C.

In general, the balance between auxin and cytokinin concentration plays an important role in differentiation (Wilson, 1991). Adding growth regulators which supporting the high rates of growth do not induce the accumulation of significant amounts of secondary metabolites (Yeoman and Yeoman, 1996).

1.5.3 Physical conditions

Plant cell and tissue cultures grow differently depending on the type of culture condition they are subjected to. The intensity, type and duration of light, temperature and physical medium composition play a role in the morphogenesis of cultures. The optimal pH medium is usually 5.5-5.8. Sometimes, pH medium can improve the uptake of nutrients and precursors, the membrane permeability and the releasing of secondary metabolites (Brodelius, 1990). Meanwhile, light might promote embryogenesis, shooting and greening of callus, and can be essential for the production of secondary metabolites. Light is usually supplied by cool-white or a special plant growth fluorescent lamp. Culture condition is often maintained around 25°C. Some species may require varying temperature treatment for optimal growth.

1.6 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 science, with significantly impact on agriculture, horticulture, pharmaceutical, food, cosmetics, and forestry. Research in plant cell and tissue culture can be divided into five 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 product, especially in the food and pharmaceutical industries (Trevor, 1990).

1.7 Aseptic technique work

All the manipulations were carried out under aseptic conditions using a laminar flow cabinet with horizontal flow of sterilised air.

1.8 Secondary metabolites

Secondary metabolites are often characteristic of a plant family. Differences in structure of secondary metabolites and their site of location in plant 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 in response to a range of physical stresses such as cold, heat, osmotic pressure and physical damage.

Therefore, secondary metabolites refer to any chemical compounds, which are not essential for the survival of plant cells, 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.

1.9 Production of secondary metabolites from plant cell and tissue cultures

Synthesis of secondary metabolites 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 synthesis cell walls and protein is converted into secondary metabolites and stored in the vacuole or cytoplasm. When more rapid growth is resumed then the secondary metabolites are degrade and the stored carbon released. The activities of secondary and primary metabolism are closely related and exist in a dynamic equilibrium linked by key enzymes. These key enzymes are very important to their activity may be a major rate-limiting factor in secondary metabolite accumulation.

Because of their complexity chemical structures, most compounds are often difficult to synthesis chemically. Furthermore, many new compounds, which are not found in the parent plants, may be produced by plant cell and tissue culture techniques.

Recently, the secondary metabolite production by plant cell and tissue techniques has been the subject of extended research. One of the problems associated with plant cell and tissue culture for the production of some particular secondary metabolites is the low yield obtained. Because of the undifferentiated cell has physiological structure different from the usual plant cell, it has no site for storage the secondary metabolites, many techniques of plant cell and tissue culture were used to improve the low yields e.g. cell immobilisation, feeding of precursor and biotransformation, permeabilisation and elicitation.

The economic production of valuable compounds from plant cell and tissue culture has been a major purpose in studying the biosynthesis of desired secondary metabolites. The selected examples with higher yield than parent plants are list in Table 1 (Dicosmo and Misawa, 1995).

According to, 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 culture have been applied for solving these problems. The selected examples of secondary metabolites, which have been studied, are Taxol, artemisinin and ginkgolide.

1.9.1. Taxol

Taxol is a complex diterpenoid alkaloid that was originally isolated from the bark extract of the Pacific yew tree, *Taxus brevifolia*. Now well known as a strong anticancer drug, the structure and biological activities of Taxol were first published in 1971 by Wani *et al.* Taxol is now a registered trademark of Bristol-Myer Squibb Co., the generic name for Taxol is paclitaxel (Seki *et al.*, 1996). The Food and Drug Administration (FDA) has approved the used of

paclitaxel for treatment of refractory ovarian cancer, recurrent and refractory metastasis breast cancer, and polycystic kidney disease (Ketchum and Gibson, 1996). The bark of *Taxus brevifolia* is the only FDA-approved source of paclitaxel for clinical used (Gibson *et al.*, 1993). Approximately 3-6 trees supply taxol (1 g) for treatment of 1 patient. Due to the relative scarcity of the tree and the low yield of taxol, the supply of taxol is restricted, limiting expansion of clinical trials and treatment availability. Continued bark harvesting for taxol does not appear to be an adequate source to meet the needs of ovarian cancer patients for 20-25 kg/year or the larger project demand for over 500 kg taxol/year to treat multiple type of cancer (Gibson *et al.*, 1993). The alternative methods for taxol production, such as from tissue culture, organic synthesis or other *Taxus* species, have been increasing. Several studies on *Taxus* plant cell culturing have been reported (Gibson *et al.*, 1993, Han *et al.*, 1994, Fett-Neto, *et al.*, 1994, Seki *et al.*, 1996, Hirasuna *et al.*, 1996, Ketchum and Gibson, 1996, and Srinivasan *et al.*, 1997). The plant cell culturing of *Taxus* species is considered another promising method to obtain Taxol and related taxane compounds. The large-scale plant cell culture may be cost-effective and enabling technology to produce taxol. The product of taxol and taxol-like compounds from tissue cultures of *T. brevifolia* has been patented by Plant, Soil, and Nutrition Laboratory, New York, USA (U.S. Patent No. 5,019504) (Gibson *et al.*, 1993).

1.9.2 Artemisinin

Artemisinin is the endoperoxide sesquiterpene lactone isolated from aerial parts of *Artemisia annua* L., a traditionally used medicinal plant from China. Artemisinin is determined as antimalarial activity, much effort is being made to develop a new class of antimalarials, based on this compound (Woerdenbag *et al.*, 1993). It has been found to be particularly active against chloroquine resistant *Plasmodium falciparum* in the treatment of cerebral malaria (Chiung-Sheue *et al.*, 1992). The highest artemisinin content has been found in leaves and flowering tops of the plant and it is influenced by several factors such as

plant variety, cultivation conditions and geographic localization (Paniego and Giulietti, 1994). On a global scale, artemisinin is hardly available, and drug registration requirements are not yet met. The total organic synthesis has been established, but is very complicated, and low yields, that are economically unattractive, are obtained. Therefore, the plant remains the only valid source of artemisinin. Alternative sources of artemisinin have been investigated from plant cell cultures (Liu, *et al.*, 1992, Woerdenbag, *et al.*, 1993, Paniego and Giulietti, 1994, Ferreira and Janick, 1996 and Kim, *et al.*, 2001).

1.9.3 Ginkgolides

Ginkgolides are unique C_{20} lactones cage molecule extracted from the leaves of the slow growing *Ginkgo biloba* L. tree. These diterpenes are formed of six five-membered rings including a spiro-nonane system, a tetrahydrofuran cycle and three lactonic groups. The synthesis of these complex products requires multi-step procedures, which are difficult to scale up economically (Carrier, *et al.*, 1991). The diterpenes ginkgolides have many pharmacological effects and are antagonists to factor that is inflammatory autacoids (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) (Huh and Staba, 1993). These phytochemicals show significant pharmacological activity against Platelet Aggregation Factor (PAF). Ginkgolide B is the most efficient PAF antagonist (Carrier, *et al.*, 1991). Plant cell culture is an interesting alternative to produce ginkgolides. Several studied of ginkgolides production on *Ginkgo biloba* cultures have been reported (Nakanishi and Habagushi, 1971, Carrier, *et al.*, 1990, Archambault *et al.*, 1990, Carrier, *et al.*, 1991 and Huh and Staba, 1993). However, the biosynthetic ability of *in vitro* growing *Ginkgo biloba* cells to produce ginkgolides must be assessed and information on the growing conditions of the cells must be gathered before proceeding to yield improvement.

Table 1 Secondary metabolites produced in high levels by plant cell and tissue cultures (Dicosmo and Misawa, 1995)

Compounds	**Plant species**	**Yield (% dry weight)**	
		Cell culture	**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
Anthraquinone	*Galium verum*	5.4 (s)	1.2
Anthraquinone	*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 culture

(s) = Cell suspension culture

1.10 Special techniques for improving the secondary metabolite production from plant cell and tissue cultures

1.10.1 Cell immobilisation

Cell immobilisation is one of the biotechnological techniques that had been introduced as a beneficial aid for the secondary metabolite production. The principle of cell immobilisation is cell 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 in phosphate, nitrate and growth regulators 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 unit for up to 30 weeks compared with 3-4 weeks for a normal cell suspension culture.

The immobilisation methods may vary but all retain the principle of providing a protected environment for the cells: (Hunter *et al.*, 1993)

1. Nylon meshes – Immobilisation can be achieved by allowing cells to adhere to sheets of nylon mesh in airlift bioreactor. Nutrients are circulated using a stream of sterile air throughout the culture.
2. Porous foam blocks – The cells grow and divide to fill the spaces in the blocks, once the spaces are filled the cells cannot grow any more and 'in theory' cellular activity is channelled away from growth processes, and hopefully, towards the synthesis of secondary metabolites.
3. Alginate beads – Along similar lines, alginate beads have been used to entrap cells. Sodium alginate is sterilised by autoclaving and cells from a suspension culture are mixed with it when cool. The cell-sodium alginate suspension is

then dropped to a solution of calcium chloride; as the two solutions mix calcium alginate beads are formed in which the plant cells become entrapped.

All these methods aim towards increasing the degree of cell-to-cell contact whilst limiting growth and maintaining a relatively easy-to-manipulate bioreactor culture. However none of these systems has yet resulted in any commercial processes and although concentrations of secondary metabolite are sometimes enhanced, they still remain well below product yield *in vivo* (Hunter *et al.*, 1993).

The advantages of immobilisation are as below: (James and Gynheung, 1986; Hulst and Tramper, 1989 and Stafford and Swarren, 1991)

1. Separation of cells from medium
2. Allowing a continuous process
3. Uncoupling of growth and production formation
4. Retention of biomass and high biomass levels
5. Reducing problems such as aggregates, wall growth, or foaming
6. Increasing metabolic stability of the plant cell
7. Stimulating the secondary metabolic production.

Selected examples of plant species, secondary metabolites and immobilisation methods have been studied in plant cell and tissue cultures are presented in table 2.

Table 2 Selected examples of plant species, secondary metabolites and immobilisation methods have been studied in plant cell and tissue cultures

Plant species	Secondary metabolites	Immobilisation methods	References
Coffea arabica	methylxanthine alkaloids	calcium alginate	Haldimann and Brodelius, 1987.
Thalictrum rugosum	berberine	agarose	Brodelius *et al.*, 1988.
Papaver somniferum	sanguinarine	surface-immobilisation bioreactor	Archambault *et al.*, 1996.

1.10.2 Feeding of precursors and biotransformation

Biotransformation is a process through which the functional groups of organic compounds are modified by living cell and is useful tool in the study of applications of plant cell culture. It is expected that specific modification of chemical structures such as epoxidation, ester formation as well as saponification, glycosylation, hydroxylation, isomerization, methylation and demethylation, reduction of double bonds, carbonyl and aldehyde functions, as well as oxidation, can be performed more easily by plant cell cultures than by micro-organism or by chemical synthesis (Reinhard and Alfermann, 1980). Now, biotransformation could be combined with immobilisation and precursor feeding and wildly used for commercial purpose.

There are some requirements for successful biotransformation of a precursor into a designated compound. These are: (Yeoman *et al*, 1990)

1. The culture must have the enzymes necessary for the transformation of precursor to product
2. The product must be formed faster than it is further metabolized
3. The culture must tolerate the added precursor and the product

4. The substrate must be able to enter the cell and the product will preferably be released into the medium.

Biotransformation of added precursors has been studied extensively to produce valuable compound. Known or putative precursors may be added to the medium or injected into plant tissue culture in order to eliminate crucial enzyme blocks or bottlenecks or to prevent compartment and effective storage of endogenous intermediates (Banthorpe, 1994). Simple intermediates have often been synthesized and converted to final compound *in vitro* with the aid of secondary product pathway enzymes extracted from plant tissue cultures and incubated with the precursor.

Selected examples of plant species, precursor feeding and biotransformation products have been studied in plant cell and tissue cultures are presented in table 3.

Table 3 Selected examples of plant species, precursor feeding and biotransformation products have been studied in plant cell and tissue cultures

Plant species	**Precursors**	**Biotransformation products**	**References**
Vitis vinifera	citral	monoterpenic alcohol, nerol and geraniol	Ambid *et al.*, 1982.
Nicotina tabacum	3-carene	3,4-eposycarene, 3-caren-5-one, 3,4-caranediol, 3,6,6-rimethylcycloheptadien-1-one, 8-hydroxy-*m*-cymene	Hirata *et al.*, 1994.
Peganum harmala	geraniol	geranyl acetate	Zhu *et al.*, 2000

1.10.3 Permeabilisation

The excretion behavior of plant cell cultures varies from one species to another, and evens 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 permeabilise 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 molecules into and out of the cell. Various methods related to permeabilisation, include treatment with solution of high ionic strength, permeabilisation with dimethylsulfoxide (DMSO) and polyoxyethylene-sorbitane 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 the organic solvent (Herman, 1993).

Permeabilisation has become a useful tool for increasing mass transfer of substrates and metabolites to and from the cells.

Selected examples of plant species, permeabilising agents and secondary metabolites have been studied in plant cell and tissue cultures in table 4.

Table 4 Selected examples of plant species, permeabilising agents and secondary metabolites have been studied in plant cell and tissue cultures

Plant species	Permeabilising agents	Secondary metabolites	References
Thalictrum rugosum *Chenopodium rubrum*	electric pulses	berberine, betacyanin	Brodelius *et al.*, 1988.
Datura innoxia	Tween 20	hyoscyamine, scopolamine	Boitel-Conti *et al.*, 1995.

1.10.4 Elicitation

Elicitation is 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). In terms of secondary product synthesis, elicitors are abiotic compounds or extracts derived from biological systems that perturb the cell culture in such a way that secondary metabolism is initiated or enhanced. Phytoalexins refer to compounds that are produced as a defence response against microbial attack. Hence, if extracts, e.g. protein or cell wall components derived from an organism known to cause disease, or attack the plant species in some other way, are introduced into the medium of the cell culture, a defence response occurs and frequently phytoalexins are produced (Hunter *et al.*, 1993).

Elicitors do not increase the yield of secondary products where the product is made for a reason other than defence. For example, the tropane alkaloids, atropine and scopolamine, are produced in nature and inhibit seeds of other plant species from germinating, so that the alkaloid producing in plant out-competes potential competitors for light, nutrients, water etc. Biotic elicitors do not enhance tropane alkaloid production. Heavy metals such vanadium have been used to elicit increased secondary metabolite production with some success in certain culture systems. This work is also empirically based because the mechanism by which heavy metals perturb plant cell culture

systems remains unknown. Only where elicitors are known to act on the whole plant *in vitro* is an analytical approach to enhancing secondary metabolite production using elicitors made possible (Hunter *et al.*, 1993).

Recently great interest has centre on jasmine and its derivatives as possible phytoalexins. Methyl jasmonate directly induces intracellular accumulation of a wide spectrum of secondary product in many plant tissue cultures (Banthorpe, 1994).

Selected examples of plant species, elicitors and secondary metabolites have been studied elicitation in plant cell and tissue culture are presented in table 5.

Table 5 Selected examples of plant species, elicitors and secondary metabolites have been studied elicitation in plant cell and tissue culture

Plant species	**Elicitors**	**Secondary metabloites**	**References**
Eschscholtzia californica	chitosan	chelerythrine, macarpine	Brodelius *et al.*, 1989.
Ruta graveolens	yeast extract	acridone epoxides, furoquinolines, furanocoumarines	Koprek *et al.*, 1992.
Artemisia annua	fungal elicitor	artemisinin	Liu *et al.*, 1999.
Petrosekinum crispum	Pep25	butylidenephthalides	Hagemeier *et al.*, 1999.

Pep25 = *Phytophthora sojae* 25-amino acid oligopeptide

Yeast extract = *Rhodotorula rubra*

Fungal elicitor = *Penicillium chysogenum*

1.10.5 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 (Banthope, 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 (Banthope, 1994).

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

The advantages of this method is secondary product 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 systems (Payne and Shuler, 1988).

Selected examples of plant species and two-phase system have been studied in plant cell and tissue cultures are presented in table 6.

Table 6 Selected examples of plant species and two-phase system have been studied in plant cell and tissue cultures

Plant species	Two-phase system	References
Vitis vinifera	Aqueous nutrient medium / Miglyol 812	Courmier and Ambid, 1987
Nicotiana tabacum	Polyethylene glycol / dextran	Hooker and Lee, 1990
Lavandula angustifolia	Aqueous nutrient medium / Miglyol 812 and n-hexadecane	Banthorpe *et al.*, 1995.

1.10.6 Cell differentiation

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. *In vitro* cultures undergo various types of differentiation, which eventually lead to changes in the metabolic events. Differentiation involving organogenesis can be visually observed and the relation between organogenesis and secondary product formation has been studies in great detail.

Cellular differentiation is another factor that influences the secondary product formation. It is therefore, evident that in a cell population, heterogeneous group of cells with different degree of differentiation occurs. While certain types of differentiations such as pigmentation are visible, the other types of differentiations such as biochemical are not visible. Various types of differentiation, which may differ from plant to plant, invariably influence the formation of plant constituents.

Transformed plant organ cultures have proved 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* and *Agrobacterium tumefaciens*. Their common mode of action is to transfer section of plasmid DNA (T-DNA) in to plant cells and to insert this T-DNA into the plant genome where it is expressed, transforming the infected plant cell. T-DNA encodes genes which modify hormone metabolism of the transformed cell and diverts it into novel routes of cellular and organ differentiation. In the case of *A. rhizogenes*, the transformed cell is induced to initiate rhizogenesis, and *A. tumefaciens* normally induces the initially transformed cells to grow in an uncontrolled manner to form callus tissue (Rhodes *et al.*, 1994).

Selected examples of plant species and secondary metabolites have been studied cell differentiation in plant cell and tissue cultures are presented in table 7.

Table 7 Selected examples of plant species and secondary metabolites have been studied cell differentiation in plant cell and tissue cultures

Plant species	**Secondary metabolites**	**References**
Rosmarinus officinalis	α-pinene, β-pinene,	Webb *et al.*, 1984
Mentha citrata	linalool, linalyl acetate	Spencer *et al.*, 1990.
Rauvofia serpentina	reserpine, ajmalicine, almaline, serpentine	Benjamin *et al.*, 1993.
Hyoscyamus muticus	hyoscyamine	Oksman-Caldentey *et al.*, 1994
Pimpinella anisum	*trans*-epoxypseudoisoeugenyl 2-methylbutyrate, geijerene, pregeijerene, zingigerene, β-bisabolene	Santos *et al.*, 1998.

1.10.7 Use of adsorbent

According to secondary metabolites especially essential oils in intact plants are accumulated in specialized secretory organs, and these can not be formed in callus or suspension cultures, so some adsorbents were used for helping collected them. The adsorption of the compounds to polymers is based on physical and chemical parameters of compounds and adsorbents. *Purge and trap* is the method refereed to the process of collecting volatile oil in an air stream by trapping them on a sorbent placed in the outlet stream, then desorbing them by the solvents. As sorption materials for trapping, a number of materials are available and used in ecological chemistry, e.g. 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 (Faldt, 2000 and Lockwood, 2001).

Zhu (Zhu, 2000) has used Porapak Q in plant tissue culture by packing it into glass tubes and collecting essential oil from callus and suspension culture. After using this technique, the collected volatiles have been increased.

1.11 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 oils, or essential oils (Tyler *et al.* 1988).

Depending on the plant family, essential oils may occur in specialized secretory structures such as glandular hairs (Labiatae), modified parenchyma cells (Piperaceae), oil-tubes called vittae (Umbelliferae), or in lysigenous or schizogenous passages (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-fertilization of flowers (Tyler *et al.* 1988).

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

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

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

1.12 Biosynthesis of essential oil constituents

The biosynthetic building blocks for terpenes are isoprene units. The so-called biosynthetically active isoprene units are isopentenyl pyrophosphate and dimethylallyl pyrophosphate, compounds that arise from acetate via mevalonic acid (Fig. 1). Geranyl pyrophosphate is C-10 precursor of the terpenes and is believed to play a key role in the formation of monoterpenes. It is formed by the condensation of one unit each of isopentenyl pyrophosphate and dimethylallyl pyrophosphate (Tyler *et al.* 1988).

As seen from Fig. 1, geranyl pyrophosphate is believed to be the direct precursor to acyclic monoterpenes. However, it must be isomerizes to neryl pyrophosphate before the cyclic monoterpenes can be formed because the *trans* isomer does not have the correct stereochemistry for cyclization. Another possibility is the formation of neryl pyrophosphate from isopentenyl pyrophosphate and dimethylallyl pyrophosphate independent of a geranyl pyrophosphate step. The intermediates in the formation of the cyclic terpenes are shown as carbonium ions; however, the true species are probably pyrophoste esters or enzyme-bound intermediate (Tyler *et al.* 1988).

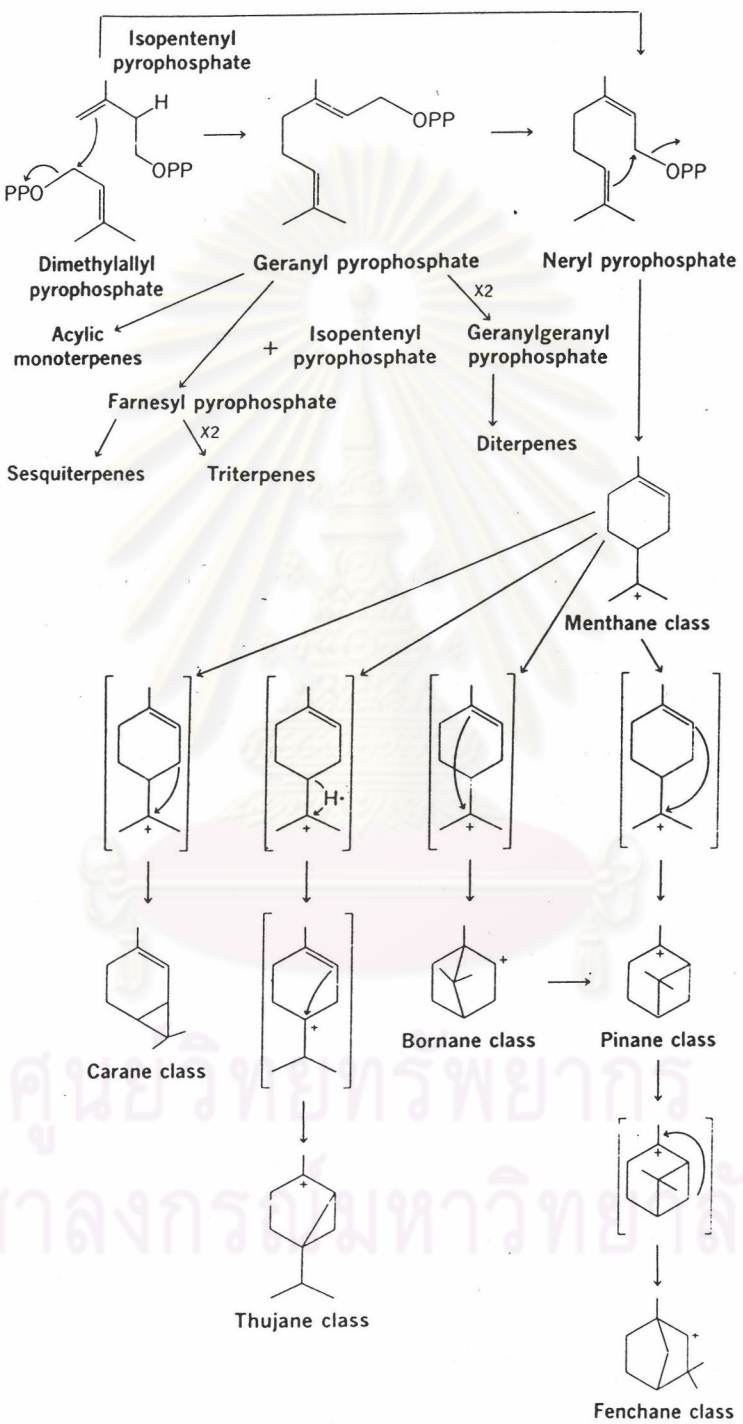

Figure 1 Biosynthetic formation of terpenes (Tyler *et al.* 1988)

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 acids phenylalanine and tyrosine, respectively, which in turn are synthesised via the shikimic acid pathway (Fig. 2). This biosynthetic pathway has been elucidated in microorganisms by using auxotropic mutants of *Escherichia coli* and *Enterobacter aerogenes* that require the aromatic amino acids for growth. In the biosynthesis, 2 glucose metabolites, erythrose 4-phosphate and phosphoenolpyruvate, react to yield a phosphorelated 7-carbon keto sugar, DAHP, this compound cyclises to 5-dehydroquinic acid, which is then converted to shikimic acid. Shikimic acid, through a series of phosphorelated intermediates, yield chorismic acid, which is an important branch-point intermediates. One branch leads to anthranillic acid and then to tryptophan. The other leads to prephenic acid, the last nonaromatic compound in the sequence. Prephenic acid can be aromatized in 2 ways. The first proceeds by dehydration and simultaneous decarboxylation to yield phenylpyruvic acid, the direct precursor of phenylalanine. The second occurs by dehydrogenation and decarboxylation to yield *p*-hydroxyphenylpyruvic 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).

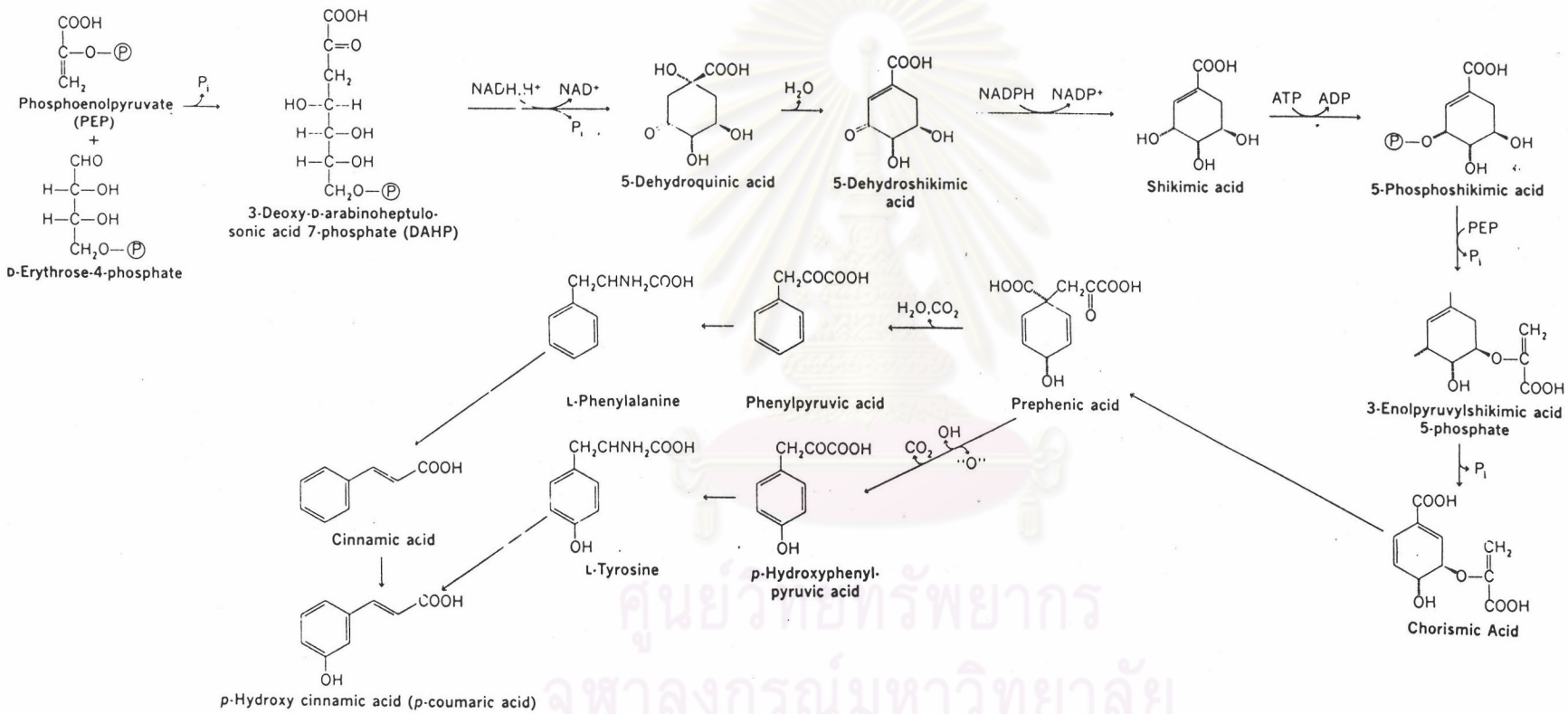

Figure 2 Biosynthesis of phenylpropanoid compounds (Tyler *et al.* 1988)

1.13 Medicinal and commercial uses

Practically all essential oils consist of chemical mixtures that are often quite which are important group of natural products with industrial interest. They have been used in perfumery and cosmetics as aroma products; in food and beverage as flavours, and as 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. eucalyptus oil), orally (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 others is used as carminatives. Oils showing antispasmodic activity, and much used in popular medicine, are those of *Melissa officinalis, Rosmarinus officinalis, Mentha piperita, Matricaria chamomile, Foeniculum vulgarae, Carum carvi* and *Citrus aurantium* (Evan, 1996).

The fabrication of perfumes is a 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 household cleaners, polishes, and insecticides (Tyler *et al.* 1988).

1.14 Production of essential oil from plant cell and tissue cultures

Plant cell and tissue cultures, one of the biotechnological methods, have been used for procurement natural raw material limitation.

Production of essential oils as secondary metabolites in plant cell and tissue cultures has proved to be difficult, and most culture 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 specialized secretory organs, and these can not be formed in callus or suspension cultures. More often, dedifferentiation of plant tissue is accompanied by a partial or 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 (Charlwood and Rhodes, 1990 and Constabel and Vasil, 1994).

It is known that monoterpenes are cytotoxic to plant tissues, causing a decrease in cell membrane permeability and inhibiting respiration and photosynthesis. Also, a significant correlation between the accumulation of essential oil and density of the storage sites has been noted (Charlwood and Charlwood, 1991).

In intact plant, toxic compounds are sequestered and storage at specialised sites, including glandular hairs, resin canals and schizogenous glands, which are not found in undifferentiated cell.

If there is any correlation between accumulation of essential oils in undifferentiated cell 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 culture will possibly be stimulated by:

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

According to this point of view, many biotechnological methods have been utilized to enhance the yield of essential oils.

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

Selected examples of essential oil production in plant cell and tissue cultures are presented in table 8.

Table 8 Selected example of essential oil production in plant cell and tissue cultures

Plant species	**Detected compounds**	**References**
Achillea millefolium	*epi*-Cubevol, neryl isovalerate	Lourenco *et al.*, 1999
Citrus sinensis	Essential oils	Niedz *et al.*, 1997
Mentha citrate, Mentha piperita	Menthone, menthofuran, menthyl acetate, menthol	Spencer *et al.*, 1993
Lavandula angustifolia	β-pinene, β-ocimene	Webb *et al.*, 1984
Origanum vulgarre spp. *virens*	Essential oils	Alves-Pereira *et al.*, 1998
Perilla frutescens	Perilladehyde	Nishizawa *et al.*, 1992
Pimpinella anisum	*trans*-Epoxypseudoisoeugenyl 2-methylbutyrate, giejerene, zingiberene, β-bisabolene	Santos *et al.*, 1998
Rosa damascena	2-Phenylethanol	Banthorpe *et al.*, 1988
Rosmarinus officinalis	α-Pinene, β-pinene	Webb *et al.*, 1984
Thymus vulgaris	Thymol	Yamaura, *et al.*,1992
Vitis vinifera	Geraniol	Cormier *et al.*, 1987