ESTABLISHMENT OF CELL SUSPENSION CULTURE OF *Hyoscyamus niger* L. FOR THE PRODUCTION OF TROPANE ALKALOIDS

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by

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For my love ones...

Bonda Radiah,

Ayahanda Ishak,

Anakanda;

Amanina, Fatin & Asyraff.

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Rafidah Ishak

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LIST OF ABBREVIATIONS

MS	Murashige and Skoog
LS	Linsmaier and Skoog
B5	Gamborg
BAP	Benzylamiopurine
NAA	Naphtaleneacetic acid
HPLC	High performance liquid chromatography
MeOH	Methanol
EtOH	Ethanol
RIA	Radioimmunoassay
GC	Gas chromatography
FID	Flame ionization detector

PEMBANGUNAN KULTUR AMPAIAN SEL *Hyocyamus niger* L. UNTUK PENGHASILAN ALKALOID TROPANA

ABSTRAK

Biji benih aseptik *Hyoscyamus niger* diperolehi melalui protokol pensterilan dua peringkat dengan menggunakan larutan Clorox® bersama Teepol dengan menghasilkan 95.6% biji benih aseptik. Dari biji benih yang aseptik, 79.8% bercambah dan 100% daripada anak benih daripada biji benih hidup. Kalus boleh diaruhkan daripada eksplan daun, petiol daun dan akar anak benih *in vitro* dengan medium MS mengandungi 2.0 - 10.0 mg/L picloram. Eksplan daun yang diinokulasi dalam medium MS mengandungi 2.0 mg/L picloram menghasilkan kalus yang lebih sesuai untuk kultur ampaian sel. Walaupun kalus yang dihasilkan daripada eksplan daun dan petiol dengan medium yang mengandungi unsur-unsur yang sama tidak berbeza ($p \leq 0.05$) dari segi kuantiti tetapi sangat berbeza dari segi kualititatif. Kalus yang dihasilkan daripada eksplan daun lebih lerai dan bergranul halus sedangkan kalus yang dihasilkan daripada eksplan petiol daun sangat padat. Kalus yang dihasilkan daripada eksplan akar menggunakan media yang sama adalah sangat sedikit, padat dan bergranul. Kalus yang terhasil daripada eksplan daun kemudian disubkultur menggunakan medium MS mengandungi 0.5 mg/L picloram menjadi lebih lerai dan kurang bergranul tapi kadar pertumbuhan adalah lebih cepat. Sel ampaian *H. niger* dihasilkan dengan memindahkan 0.5 g kalus yang paling lerai dan halus yang terhasil daripada daun dimasukkan ke dalam 25 mL medium MS cair mengandungi 2.0 mg/L picloram. Selepas pengoptimuman medium proliferasi sel yang paling baik adalah medium MS mengandungi 0.5 mg/L picloram, 30 g/L sukrosa dan 0.1 mg/L myo-inositol, dengan tempoh masa sub-kultur ditetapkan selama empat belas hari. Pola pertumbuhan *H. niger* berdasarkan jisim sel basah dan

kering adalah mengikut lengkung sigmoid yang tipikal. Elisitasi dilakukan dengan menambah amaun kalsium klorida, ekstrak yis dan kasein hidrolisat yang berbeza kepada medium. Kepekatan kalsium klorida yang lebih rendah menyebabkan kadar pertumbuhan sel yang lebih tinggi berbanding dengan kalsium klorida kepekatan yang tinggi. Penambahan kalsium klorida tidak mempengaruhi penghasilan hiosiamin tetapi meningkatkan penghasilan skopolamina bila kepekatan kalsium klorida adalah dua kali ganda kandungan di dalam medium MS normal (0.44 b/L). Elisitasi dengan 3.0 g/L estrak yis menyebabkan kadar pertumbuhan sel yang lebih rendah sedangkan elisitasi dengan 2.0 g/L ekstrak yis menyebabkan peningkatan ketara dalam penghasilan hiosiamin dan skopolamina dalam kultur ampaian sel *H. niger*. Kandungan hiosiamin dan skopolamina yang dihasilkan berkurang apabila kepekatan estrak yis ditambah sehingga 3.0 mg/L. Elisitasi dengan kasein hidrolisat tidak mempunyai kesan yang ketara terhadap kadar pertumbuhan sel *H. niger* mahupun penghasilan hiosiamin dan skopolamina.

ESTABLISHMENT OF CELL SUSPENSION CULTURE OF *Hyoscyamus niger* L. FOR THE PRODUCTION OF TROPANE ALKALOIDS

ABSTRACT

Aseptic seeds of *Hyoscyamus niger* were established via two-stage surface sterilization protocol using Clorox® solution coupled with Teepol with 95.6% of the seeds were aseptic. From these aseptic seeds, 79.8% of the seeds germinated and 100% of the seed-derived plantlets survived. Callus could be induced from the leaf, petiole and root explants of the *in vitro* plantlets on MS medium supplemented with 2.0 - 10.0 mg/L picloram. The leaf explants inoculated to MS medium supplemented with 2.0 mg/L picloram produced the best callus for cell suspension cultures. Even though the callus produced by the leaf and the petiole explants on the same medium was not significantly different ($p \leq 0.05$) in terms of quantity, they are very different qualitatively. The callus produced by the leaf explants was more friable with tiny granules while the callus produced by the petiole explants was very compact. Small amount of compact and granular callus was produced from the root explants on the same medium. The leaf-derived callus subsequently sub-cultured on MS medium supplemented with 0.5 mg/L picloram became more friable with less granules but with higher growth rate. The cell suspension culture of *H. niger* was initiated by transferring 0.5 g of the most friable portion of the leaf-derived callus into 25 mL MS liquid medium supplemented with 2.0 mg/L picloram. After optimization, the best cell proliferation medium was MS medium supplemented with 0.5 mg/L picloram, 30 g/L sucrose and 0.1 mg/L myo-inositol, with the sub-culture time period fixed at fourteen days. The growth pattern of *H. niger* based on fresh and dried cell mass followed a typical sigmoid curve. Elicitations were carried out by adding varying amount of calcium chloride, yeast extract and casein hydrolysate into the

medium. Lower concentration of calcium chloride induced higher cell growth as compared to higher concentration of calcium chloride. The addition of calcium chloride into the cell culture medium did not affect the production of hyoscyamine but, the production of scopolamine increased when the concentration of calcium chloride was doubled from the normal MS concentration (0.44 g/L). Elicitation with 3.0 g/L of yeast caused lower cell growth while elicitation with 2.0 g/L yeast extracts caused significant increased in the production of hyoscyamine and scopolamine in the cell suspension cultures of *H. niger*. The content of hyoscyamine and scopolamine produced decreases when the concentration of the added yeast extract was increased up to 3.0 mg/L. Elicitation with casein hydrolysate did not have any significant effect on the growth of *H. niger* cells nor the production of hyoscyamine and scopolamine.

CHAPTER ONE

INTRODUCTION

Plants have always played an important role in human life. Besides being the major resources for human basic needs, plants also synthesize and preserve a variety of biochemical products, many of which are extractable to be used in cosmetics and pharmaceutical industries. Over 80% of the approximately 30,000 known natural products are of plant origin (Rao and Ravishankar, 2002; Phillipson, 1990). In pharmaceuticals, certain plant products are irreplaceable because of their therapeutic properties. Throughout the world, 121 prescription drugs are identified as plant derived drugs (Payne, 1991).

Most plant natural products used in pharmaceuticals are derived from the secondary metabolites of plants. Plants produce a variety of economically important secondary metabolites which are being used as pharmaceuticals, antioxidants, insecticides and food additives. Over 100,000 secondary metabolites are known compounds, and about 4000 new compounds are being discovered every year (Zhang *et al.*, 2002). Several widely known secondary metabolites are phenylpropanoids, alkaloids, terpenoids, quinines and steroids (Rao and Ravishankar, 2002).

Alkaloids have been used in traditional medicine since ancient time and in modern medicine for as long as it has been discovered. Plants have been a unique source of therapeutically significant alkaloids. Within the plant kingdom, angiosperms are the major source of alkaloids and have always been the important sources of drugs. Alkaloids are the most diverse group of plant secondary

metabolites and currently there are over 12,000 known alkaloids. The list is growing as more alkaloids are being discovered each year. The earliest documented references to Solanaceous plants, the main angiosperms source of alkaloids, dated from at least 2000 B.C. This proves that the powerful pharmacological properties of *Hyoscyamus niger*, *Mandragora officinarum* and *Atropa belladonna* were already known since ancient Egypt and Mesopotamia (Christen, 2002).

Hyoscyamus niger is one of the important sources of the tropane alkaloids, hyoscyamine, scopolamine and atropine. Bellamy and Pfister (1992), in "World Medicine: Plants, Patients and People", include *Hyoscyamus niger* in their summary of "Planet Earth's materia medica" list of "The Family of Healing Plants". According to the list, *Hyoscyamus niger* had been used as antispasmodic, to counteract spasm and griping action of purgatives, as cerebral and spinal sedative, to treat insomnia, to relief cystitis pain and as domestic remedy for toothache. The sources of the list are the 1988 edition of British Pharmacopoeia, the 1979 and 1907 editions of British Pharmacopoeia Codex and the 1966 edition of the first Book of Avicenna's Canon as used in Unani Medicine.

The tropane alkaloids, hyoscyamine, scopolamine and atropine, are in fact important substances in modern medicine. However, since the sources of these alkaloids are temperate plants, these plants cannot be cultivated in tropical countries like Malaysia. Consequently, the pharmaceutical companies in Malaysia have to import the dried plant parts to be used in the industry.

Plant cell culture technique which is independent of geographical and seasonal variation is an attractive solution to this problem. This technique has

been used for other plant species. Plant cells are biosynthetically totipotent, which means that each cell in the culture retains complete genetic information of the parent plant. Thus, cell culture is capable of producing the same range of chemicals found in the parent plant (Rao and Ravishankar, 2002). As a result, as long as the *in vitro* culture system of the cells can be established, the relevant chemicals needed can be produced anywhere regardless of the climate difference.

This study was carried out to explore the possibility of establishing an optimal *in vitro* cell culture of *Hyoscyamus niger* L. as an alternative source of the alkaloids. Consequently, the objectives of this study are:

1. To establish cell suspension culture of *Hyoscyamus niger*
2. To optimize the culture system for maximum growth of *Hyoscyamus niger* cells and the production of tropane alkaloids in the cell suspension culture of *Hyoscyamus niger*
3. To study the effect of elicitors on the production of tropane alkaloids in *Hyoscyamus niger* cell suspension culture.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Hyoscyamus Niger*

2.1.1 Plant origin and biography

Hyoscyamus niger is a European native of Solanaceae family. Its common name is black henbane. Originally, the old Anglo Saxon name for it was ‘Belene’, presumably due to its bell-shaped flowers. However, since its poisonous properties were recognized this name was changed to ‘Henbane’ (Grieve, 2006). The name henbane is originated from the Anglo-Saxon terms, ‘henn’ means chicken and ‘bana’ means murderer. This foul smelling weed is called the chicken murderer because when the fowls eat the seeds of this plant they become paralysed and die. The seeds are also believed to be poisonous to children, rodents, and fish (Haas, 1995). However, it was discovered that the pig could eat it with impunity. Due to this reason, the herb is sometimes referred to as hog bean. In fact, its botanical name *Hyoscyamus* and the tenth-century *Jusquiasmus* are derived from the Greek words *hyos* which means ‘hog’ and *kyamos*, which means ‘bean’ (Panda, 2000). Other names used to refer to this herb are *Hyoscyami folium*, devil's eye, foetid nightshade, henbell (Anglo-Saxon), Jupiter's bean, poison tobacco, stinking nightshade, symphonica, cassilato and cassilago (Haughton, 2010).

Hyoscyamus niger originally found around the Mediterranean basin, but has been widely distributed in Eurasia (Roberts and Wink, 1998). This plant, despites the negative connotation of its poisonous properties, is very important in pharmaceuticals. Due to its importance in the modern medicine, the herb is cultivated

in Europe and North America for drug use (Dewick, 2002). Easily grow in dry soils, this herb shrub has scattered throughout the waste area and roadsides in Southern Canada, Northern United States of America and commonly found in some areas of the northern Rocky Mountain states (Beasley, 1999).

2.1.2 Plant morphology

Hyoscyamus niger L. grows up to 4 feet tall. The leaves are large, pale green and oval shaped, 2 – 3 inches in length with deeply toothed edges and covered with sticky hairs. The flowers are single on short pedicels, bell-shaped, five lobed, with mustard-yellow petals and purplish-brown throats and veins. They are hermaphrodite flowers which are pollinated by insects (Beasley, 1999). The fruits are oblong berries, about 1 cm long, enclosed in 5 lobed capsules. In the berries are numerous tiny seeds, brown to black in colour (Kurian and Sankar, 2007).

The plant has both annual and biennial forms. Both forms spring indifferently from the same crop of seeds but mature differently. The annual form has simple downy stem, toothed sinuate leaves and sessile flowers. It flowers in July and August. The biennial form is larger, stronger, has more branches and more odorous. During the first year, it produces only a tuft of radical leaves with no aerial stem. In the autumn, the leaves die but the biennial root survives the winter. Later in spring it forms a flowering stem from the underground roots which grows up to four feet tall and branches vigorously. The leaves are large and deeply sinuate. It flowers in May or June. Both forms can be used in medicine, but the biennial form is believed to posses more medicinal properties (Grieve, 2006).

2.1.3 Chemical Properties and Medicinal Uses of *Hyoscyamus niger*

All parts of the plant are highly poisonous. Even small doses can be sedative and hallucinogenic, while large doses can be fatal. This plant is so dangerous that its poisonous principal can absorb into human body through skin (Bellamy, 1992). Small ingestion of any part of the plant can cause dizziness to delirium along with other anti-cholinergic effects. John Gerard (1545-1611) the renown herbalist, described henbane poisoning as akin to alcohol poisoning which caused stupor followed by comatose sleep (Haas, 1995). In fact, henbane extracts had been used in beer brewing, as substitute for hops due to its bitter taste and the ability to enforce the effects of ethanol. However modern laws in many countries had banned the addition of the tropane alkaloid plant extracts in beer brewing (Roberts and Wink, 1998).

The poisonous property of this plant is due to the presence of narcotic tropane alkaloids, hyoscyamine, scopolamine and atropine, which are produced as secondary metabolites. Scopolamine constitutes about 40% of total alkaloids in the plant. The presence of these alkaloids contributes to the hallucinogenic, analgesic, and toxic effects of this plant. As a consequence, *H. niger* is one of the infamous herbs used by witches and magicians during ancient Greece and Romans. In modern days, its alkaloids are used as sedative before surgery, in ophthalmology and to prevent seasickness (Roberts and Wink, 1998).

H. niger medicinal history started since ancient time. Due to its hallucinogenic property, *H. niger* (Henbane) along with other Solanaceae plants such as *Atropa belladonna* (Deadly Nightshade), *Mandroga officinarum* (Mandrake) and *Datura stramonium* (Thorn-Apple) were all used by witches, probably since they

were first discovered. It is believed that the flight of the broomstick witches was probably a flight through the mind caused by the hallucinogenic property of these plants (Bellamy, 1992; Roberts and Wink, 1998).

The hallucinogenic property of *H. niger* is mainly due to the presence of tropane alkaloids in the plant. Tropane alkaloids are compounds known as muscarinic receptor antagonists. These substances interfere with the parasympathetic nervous system because they chemically resemble the neurotransmitter acetylcholine (Börsch-Haubold, 2007). These compounds bind to the muscarinic receptor of the synapse thus block the action of the neurotransmitter acetylcholine on post-ganglionic cholinergic nerves of the parasympathetic nervous system essentially by blocking its binding to muscarinic cholinergic receptors (Christen, 2002). This blocking leads to an inhibition of sweat and saliva production and to relaxation of smooth muscles, especially those of stomach and intestine. Hyoscyamine can pass through the blood-brain barrier by diffusion, thus the central nervous system is also affected (Roberts and Wink, 1998).

At low doses (0.5-1.0 mg), atropine leads to mild excitation, whereas scopolamine causes drowsiness, fatigue, dreamless sleep and euphoria. The ingestion of higher doses causes restlessness and hallucinations. Hyoscyamus poisoning with about 10 mg of atropine (less for children) leads to central depression of life functions, which may progress to coma, circulatory collapse and respiratory failure (Börsch-Haubold, 2007).

Traditionally, *H. niger* had been used as narcotic, sedative and antispasmodic in treating internal organ pain including uterine pain, intestinal colic, haemorrhoid pain, urinary irritation, gout, rheumatism, ear-ache and headache. It was also

considered useful for mania, restlessness, nervous excitability, delirium and nymphomania. Its antispasmodic properties made it useful in the treatment of chronic choughs, bronchitis, pneumonia, and asthma (Foster and Hobbs, 2002)

In modern medicine, the applications of tropane alkaloids in pharmaceutical include powerful bronchodilators to treat chronic bronchitis, as midriatics for dilation of pupil of the eye to facilitate optical surgery and as antimuscarinic drugs to control Parkinson's disease (De Luca and St Pierre, 2000). Its anti-spasmodic property enables *H.niger* to be used in treating the symptoms of Parkinson's disease by relieving the tremor and rigidity during the early stages of the illness. *H. niger* has also been used as a sedative and pain killer especially to relieve pain of the urinary tract such as kidney stones and abdominal cramping. To treat asthma and bronchitis, this herb has been used in the form of a cigarette while the oil has been used externally applied for neuralgia, sciatica, and rheumatism. In homeopathic medicine, *H. niger* is used for dry, spasmodic coughs, epilepsy, and typhoid fever or typhoid pneumonia (Nash, 2005).

2.2 Secondary Metabolites

2.2.1 History and definition

Secondary metabolites are compounds that are not involved in the primary events essential for plant survival. Even though these substances are not involved in primary plant metabolism, they are nevertheless important in plant survival. More than one hundred years ago, in 1888, Ernst Stahl (1848-1919, Jena, Germany) had proven experimentally that secondary metabolites serve as defence compounds against snails and other herbivores. Beside Stahl, two other protagonists of chemical

ecology of the nineteenth century shared the same believe that secondary metabolites are actually a chemical defence mechanisms in plants. They are Anton Kerner von Marilaun (1831-1898, Innsbruck and Vienna, Austria) who studied the impact of geological, climatic and biotic factors on plant survival, and Lëo Errera (1858-1906, Brussels, Belgium) who analysed the localization of the alkaloids in plant tissues (Hartman, 2007). However this hypothesis was not accepted by most botanists during that time and in general they regarded secondary metabolites as waste products of primary metabolism (Wink, 2003; Hartman 2008). In contrast to the hypothesis, the difference in the concept of secondary metabolites as opposed to primary metabolites was first defined by Albrecht Kossel in 1891. He stated that, as primary metabolites are present in every living plant cell that is capable of dividing, secondary metabolites are present only 'accidentally' and are not essential for plant life (Rhodes, 1994 and Edreva *et al.*, 2008). Thirty years later in 1991, Czapek made an important step forward by dedicating an entire volume of his 'Plant Biochemistry' series to these metabolites which he named 'end product'. He described secondary metabolites as the products of nitrogen metabolism through secondary modification such as deamination (Bourgaud *et al.*, 2001).

Interest in plant secondary metabolites has risen dramatically in recent years among plant molecular biologists and plant breeders. The term 'secondary' metabolite is rather misleading. As mentioned, it portrays the negative connotation as if it is the product of mistaken primary metabolism and of little importance to the plant metabolism and growth. Studies have proven that many secondary metabolites are in fact the key components of active and potent plant defence mechanism to fight against their pest and pathogens (Bennet and Wallsgrove, 1994). Relevant to these discoveries, in addition to plant secondary compounds, the terms phytochemicals,

antinutritional factors, and plant xenobiotics have also been used in the literature to refer to this group of compounds (Makkar *et al*., 2007).

Today it is widely accepted that secondary metabolites are produced by plants for defence mechanism, to inhibit or kill their enemy such as herbivores and microbes. Roberts and Wink (1998) and Wink (2003) described the bitter taste of many alkaloids, one of the most important secondary metabolites, in plants as feeding deterrent to animals, thus inhibit the plant consumption by animals. Hence, plants containing secondary metabolites particularly alkaloids, saponins and tannins are generally avoided by grazing animals and leaf feeding insects (Kayani *et al.*, 2007).

Secondary metabolites also serve as signal compounds to attract pollinating and seed dispersing animals. Some secondary metabolites carry out physiological functions, such as mobile and toxic nitrogen transport and storage compounds (alkaloids and peptides) or as UV-protectants (phenolic compounds) (Wink, 2003).

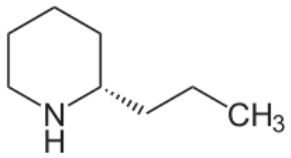
2.2.2 Types of Secondary Metabolites

2.2.2.1 Alkaloids

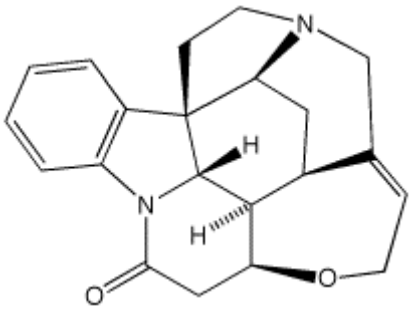
Alkaloids are organic nitrogenous bases, containing one or more nitrogen atoms, as primary, secondary or tertiary amines. The name alkaloid is derived from the term alkali. However, the basicity of the alkaloids varies greatly, depending on the structure of the molecule and the location of the functional groups present. In fact, some alkaloids are essentially neutral (Dewick, 2002).

The term alkaloid was first mentioned in 1819 by W. Meißner, an apothecary from Halle who observed the like-alkali properties of the compound thus named it alkaloid. However, the complete definition of the term is rather complex due to its similarities to other secondary metabolites and with certain exceptions in many groups. Generally, most biologist define alkaloid as a pure and perfect natural product that is biologically active and heterocyclic, nitrogen containing chemical compound which may have pharmacological properties and medicinally or ecologically useful. The medical scientist define the term alkaloids as any group of nitrogenous substances of vegetable origin with complex heterocycle structure containing primary, secondary or tertiary base, or quaternary ammonium groups which occur in animals and plants and has pharmacological effects on humans and animals. Chemists define alkaloids as biogenic, nitrogen containing and mostly N-heterocyclic compounds, except amino acids, peptides, nucleosides, amino sugar and antibiotics (Aniszewski, 2007).

Even though there is no one completely satisfactory definition of alkaloid, in general the alkaloids can be defined as a diverse group of organic basic substances containing secondary, tertiary or cyclic amines. Alkaloid group is very heterogenous, ranging from simple compounds such as coniine of the hemlock, *Conium maculatum* L., to the pentacyclic structure such as strychnine of the *Strychnos nux-vomica* L. bark (Figure 2.1). Alkaloids are often toxic to humans, with dramatic physiological and neurological activities (Makkar *et al*., 2007)



Coniine ($C_8H_{17}N$)



Strychnine ($C_{21}H_{22}N_2O_2$)

Figure 2.1: The contrast structures of coniine and strychnine (Lewis, 2003)

Alkaloids are divided into three major classes depending on their precursors and final structures. The classes are true alkaloids, pseudoalkaloids and protoalkaloids. The true alkaloids are bases, derived from amino acids and contain nitrogen in the heterocyclic rings. Examples are nicotine and atropine. The pseudoalkaloids are bases but not derived from amino acids. Examples are caffeine and solanidine. The protoalkaloids are bases, derived from amino acids and contain nitrogen, but not in the heterocyclic rings. Examples are the phenylethylamine derived alkaloids such as mescaline. The alkaloids are often unevenly distributed in the plant families. Common alkaloid containing plants can be found in the Leguminosae, the Liliaceae, the Solanaceae and the Amaryllidaceae. For the Papaveracea, all genera studied contained at least one alkaloid (Bennet and Wallsgrove, 1994)

Alkaloids can be found in about 20% of plant species. Many of the approximately 12,000 alkaloids for which structures have been described, function in the defence of plants against herbivores and pathogens. The potent biological activity of some alkaloids has also led to their exploitation as pharmaceuticals, stimulants, narcotics, and poisons. Plant-derived alkaloids currently in clinical use include morphine and codeine, as analgesics, vinblastine and taxol, as anticancer agents, colchicine, as gout suppressant, (+)-tubocurarine, as muscle relaxant, ajmaline, as antiarrythmic, sanguinarine, as antibiotic, and scopolamine as sedative. Other important alkaloids of plant origin include caffeine, nicotine, cocaine, and heroin, the synthetic *O,O*-acetylated morphine derivative (Facchini, 2001).

2.2.2.2 Tropane Alkaloids

The tropane alkaloids are a well-recognized group of structurally related natural products having in common the azabicyclo[3.2.1] octane structure giving systematic name for the group as 8-methyl-8 azabicyclo[3.2.1] octane (Christen, 2002). Now it is well established that the tropane ring system derives its pyrrolidine ring from ornithine or arginine or both and *N*-methyl-Δ^1-pyrrolidine salt is the common intermediate. Most of the alkaloids are mono-, di- and tri-esters of hydroxytropanes with various organic acids. Hyoscyamine and scopolamine are important representatives of tropane alkaloids (Christen et al., 2008).

Tropane alkaloids can be found in the plants from Convolvulaceae, Proteaceae, Rhizophoraceae, Erythroxylaceae, Olacaceae, Euphorbiaceae and Brassicaceae families. However, as listed in Table 2.1, Solanaceae plants are the main sources of these alkaloids. Within the Solanaceae family, Datura and Hyoscyamus genera hold the highest number of species with many different types of alkaloids, while among the popular sources of tropane alkaloids within the Solanaceous species include *Atropa belladonna* (deadly nightshade), *H. niger* (henbane) and several other *Hyoscyamus* species, *Datura stramonium* (thornapple) and other *Datura* species, and *Duboisa* species. These alkaloids are also responsible for the pronounced toxic properties of these plants (Dewick, 2002).

The tropane alkaloids hyoscyamine, scopolamine/hyoscine and cocaine are among the most important of the natural alkaloids used in medicine. Scopolamine and hyoscyamine were prominent since the Middle Ages when they were used as hallucinogens. Scopolamine remains significant in modern medicine as premedication administered before surgery under general anaesthetic. Atropine (the

racemic of hyoscyamine), causes dilation of eye pupils at 1 in 130,000 parts of water. During the Renaissance, fashionable ladies would drop belladonna extract (containing atropine) into their eyes to make themselves appear more attractive. Nowadays atropine is widely used in ophthalmic as pupil dilating agent. Cocaine which was first isolated from the leaves of Peruvian *Erythroxylon coca* plant was well known for its local anaesthetic properties. Its ability to stimulate the central nervous system and improves physical endurance led to the drug being widely administered in Europe in various medications, before its addictive properties were fully realised (Humphrey and O'Hagan, 2001).

H. niger is one of the major sources of the tropane alkaloids hyoscyamine, scopolamine (also known as hyoscine) and atropine (Figure 2.2) which are among the most important natural alkaloids used in traditional and modern medicine.

l-Hyoscyamine, was first isolated by Geiger in 1833 from henbane (*H. niger*). Currently, the major source of *l*-hyoscyamine is *H. muticus* L., indigenous to Egypt, but now cultivated in southern California. Hyoscyamine however is rarely isolated. Instead, it is usually racemized during the isolation process to atropine. Atropine, the optically inactive form of *l*-hyoscyamine, was first isolated by Mein in 1833 from *Atropa belladonna* while the scopolamine was first obtained by Ladenburg in 1881 from *H. muticus*. The salt of atropine and *l*-hyoscyamine are affected by light, moisture and heat. Scopolamine which has higher pharmacological values is the epoxide of hyoscyamine . Hyoscyamine conversion to scopolamine is catalysed by Hyoscyamine-6-hydroylase (H6H), a 2-oxoglutarate-dependent dioxygenase enzyme. The metabolic pathway of tropane alkaloids was determined as in Figure 2.3 (Mizukami and Hayashi, 2010; Cordell, 1981).

Table 2.1: Distribution of Tropane Alkaloids in the Plant Kingdom (Dewick, 2002).

Families	Genera containing tropane derivatives	Number of species containing tropane	Approximate number of alkaloids
Euphorbiaceae	*Phyllyantus*	1	1
Brassicaceae	*Cochlearia*	1	1
Proteaceae	*Agastachys*	1	2
	Bellendena	1	10
	Darlingia	2	7
	Knightia	2	18
Rhizophoraceae	*Bruguiera*	3	7
	Crossostylis	3	6
	Pellacalyx	1	1
Erythroxylaceae	*Erythroxylum*	35	78
Olacaceae	*Heisteria*	1	1
Solanaceae	*Anthocercis*	9	23
	Anthotroche	3	7
	Atropa	6	23
	Crenidium	1	9
	Cyphanthera	7	22
	Cyphomandra	1	5
	Datura	14	62
	Duboisa	3	22
	Grammosolen	1	7
	Hyoscyamus	12	27
	Latua	2	3
	Mandragora	3	13
	Nicanra	1	1
	Physalis	2	7
	Physochlaina	6	13
	Przewalskia	2	6
	Salpichroa	1	3
	Schizanthus	5	20
	Scopolia	9	16
	Solandra	6	13
	Symonanthus	1	8
	Withania	1	3
Convolvulaceae	*Calystegia*	1	3
	Colutea	1	1
	Convolvulus	5	14
	Erycibe	3	3
	Evolvulus	1	3

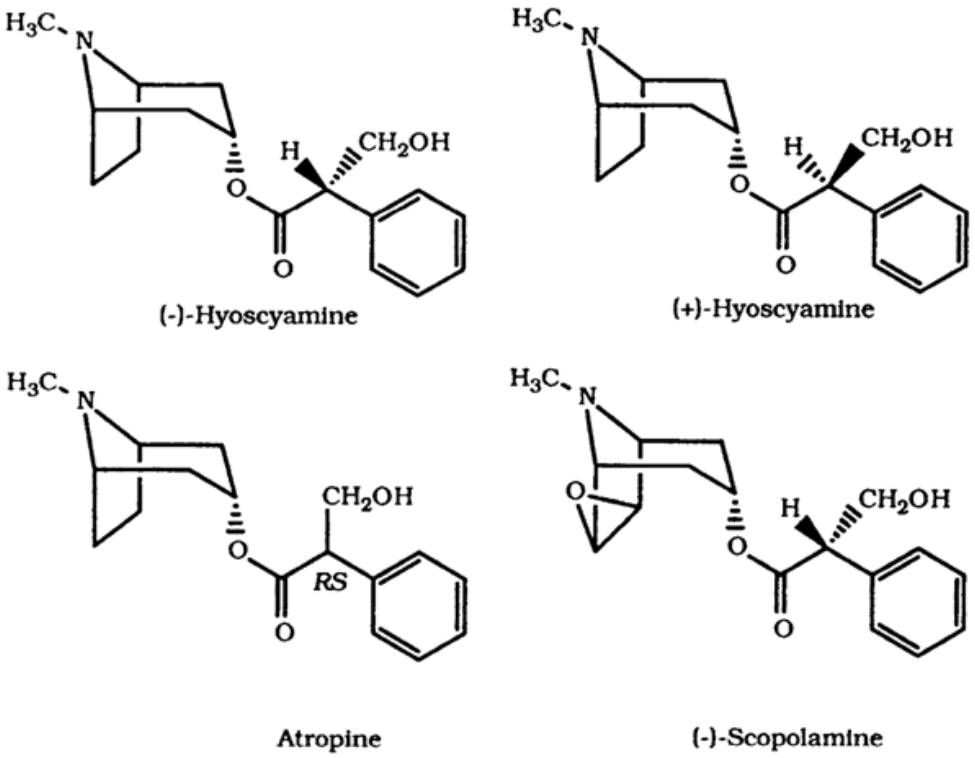


Figure 2.2: The chemical structures and formulas of hyoscyamine[$C_{17}H_{23}NO_3$], atropine[$C_{17}H_{23}NO_3$] and scopolamine[$C_{17}H_{21}NO_4$] (Christen, 2002)

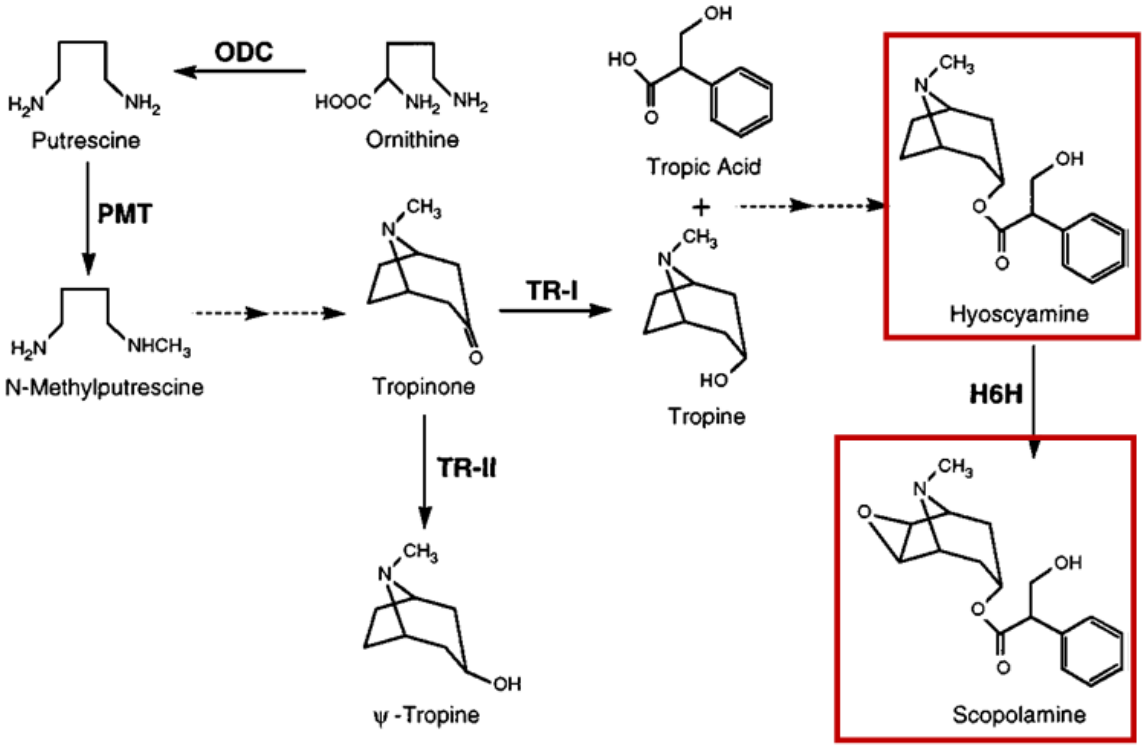


Figure 2.3: Metabolic pathway of tropane alkaloids in *Hyoscyamus niger* (Cordell, 1981)

2.3 *In Vitro* Plant Culture Technology

2.3.1 Theory and History

The term plant tissue culture is generally used for the aseptic culture of cells, tissues, organs and their components, under defined *in vitro* physical and chemical conditions (Smith, 2000). The principles of tissue culture were originated from the cellular theory introduced by Schleiden and Schwann in 1838-1839. The theory implicitly postulated that the cell is capable of autonomy and totipotency (the latent capacity of a cell to produce a whole new plant). The theory was later demonstrated and described in details by Trécul in 1833 and by Vöchting in 1878, who observed callus development. In 1893, Rechinger investigated experimentally the minimum limit of divisibility of plant parts on moist sand. He concluded that the minimum size of explants capable to divide was 1.5mm (Gautheret, 1982).

In 1898, Haberlandt began to verify the cell theory experimentally and published his results in 1902. He used single cell of palisade tissue, pith tissue, glandular hairs and stamen hairs of *Tradescantia spathacea* on Knop's solution supplemented with sucrose, aspargine and peptone. The cells used stayed alive for 20-27 days, increased in volume, but did not divide. This failure, not correctly explained at that time, is now understood to be due to the lack of 3-indole-acetic acid in the Knop's solution he used as culture medium. Even though 3-indole-acetic acid had been discovered 13 years earlier by the chemist Salkowski, its cell-dividing property was only discovered 31 years later. Later, Haberlandt indirectly studied tissue culture through wound healing and concluded that cell division was controlled by two plant hormones. One associated with the vascular system, designated as 'leptohormone', and the other was wound hormone secreted by injured cells. This

finding later became a kick start theory to the modern research on this field (Dodds and Roberts, 1985; Gautheret, 1982).

By predicting that one could successfully cultivate artificial embryo from vegetative cells, Haberlandt had established the concept of totipotency. On the basis of his address to the German Academy of Science in 1902 of his experiments on the culture of single cells, as mentioned above, and his other pioneering experimentations before and later, Gottlieb Haberlandt was justifiably recognized as 'the father of plant tissue culture'. (Thorpe, 2005; Dodds and Roberts, 1985).

In 1939, the first true plant tissue culture works were being published by Gautheret, Nobécourt and White independently, almost simultaneously. Gautheret and Nobécourt worked on the normal cambial tissue of carrot root which required auxin while White worked on tumor tissue of tobacco which did not require auxin (Gautheret, 1982).

Plant tissue culture is currently an important technique in the study of plants, either for improvement of the plant quality in agriculture or to enhance the production of the plant secondary products in pharmaceuticals. In the last few years, more discoveries have been made of the cell cultures which are capable of producing medicinal compounds at a similar rate or better than the intact plants (Vanisree *et al*., 2004).

Vanisree *et al*. (2004) listed five major advantages of the cell culture system over the conventional cultivation of whole plants. First; the useful compounds can be produced under controlled conditions independent of climatic changes or soil conditions. Second; the cultured cells would be free of microbes and insects. Third;

the cells of any plants, tropical or alpine, could easily be multiplied to yield their specific metabolites. Fourth; automated control of cell growth and regulation of metabolite processes would reduce the labour costs and improve productivity. And fifth; organic substances are extractable from the cell cultures.

Relatively, Mantell and Smith (1983) and Rao and Ravishankar (2002) highlighted fives reasons to using plant tissue culture. First, the world population is increasing rapidly, thus there is extreme pressure on the available cultivable land. Second, tissue culture is independent from various environmental factors such as climate and geographical factors. Third, it provides defined production systems for consistent product quality and yield. Fourth, it reduces the uses of land for 'cash crops', and fifth, the freedom from political interference.

2.3.2 Callus Culture

Callus is a lump of undifferentiated or unorganised cell masses, forms from the uncoordinated and disorganised growth of small pieces of plant tissue, the explants. The explants are pieces of tissue or small organs obtained from a whole plant.

For initiation of callus, a wide range of plant organs and tissues can be used as a source of explants. The sources of the explants depend on the type of culture to be initiated, the purpose of the culture and the species involved. Several factors to be considered in the selection of the explants are; the age of the explants source, the season in which the explants are obtained, the size and parts of the explants on the source plants, quality of the source plants and the purpose of the callus culture. These factors will contribute to the ability of the explants to produce actively

dividing, healthy and free from contamination callus tissue (George *et al*., 2008; Smith, 2000).

When an explants is placed on a growth supporting medium supplemented with growth regulators under aseptic conditions, the differentiated cells revert to form new meristematic tissue consist of undifferentiated cells. This process is named dedifferentiation, and as a result a coherent and amorphous tissue named callus is formed (George *et al*. 2008).

The medium for callus culture should include major inorganic nutrients, trace elements, iron source, vitamins, carbon source and plant growth regulators. The type of plant growth regulators are used according to the needs of specific culture. Auxin helps to maintain the growth of the dedifferentiated cells and cytokinin promotes cell division. Both auxin and cytokinin are needed for callus growth but in some cases, higher concentration of auxin is needed for callus initiation than for callus growth. Synthetic auxins and cytokinins are more commonly used in plant cell culture work as compare to the naturally occurring ones. For example, synthetic 2,4-D and NAA have largely replaced the natural auxin IAA while BAP and kinetin are the most commonly used cytokinins instead of the natural GA_3 (Dixon and Gonzales, 1994).

For induction of callus culture, solid and semi-solid medium are widely used. Agar, agarose or Gelrite can be used to solidify the medium. Agar is the most commonly used gelling agent because when mixed with water agar melts at 60 – 100^{o}C and solidifies approximately 45^{o}C. This means that the gels are stable at all incubation temperature. In addition, agar gels do not react with medium constituents and are not digested by plant enzymes, thus do not affect the medium or the culture (Torres, 1989).

2.3.3 Cell Suspension Culture

Cell suspension culture is a very convenient method for mass proliferation of cells and large scale production of secondary compounds. The cell suspension culture system can be optimised to achieve adequate growth rates for efficient production of the desired compounds (Razdan, 2003).

Plant cell suspension culture is usually applied for three main purposes: for the production of secondary metabolites, micropropagation and the study of plant genetics, physiology, biochemistry and pathology. As a method for producing the secondary metabolites, plant cell culture has advantages because it is not limited by environmental, ecological or climatic conditions, thus the cells can proliferate at higher growth rate than whole plants. Some plant cell cultures produce even higher concentration of the secondary metabolites compared to the parent plants thus making it more commercially viable for production of high-value added plant-specific metabolites (Zhong, 2001).

Cell suspension culture is usually initiated by transferring an inoculum of friable callus into liquid medium, and then agitate it on an orbital platform shaker at 100 – 150 rpm. The liquid medium is usually similar in composition to the callus culture medium with some adjustments being made to the concentrations of the plant growth regulators and the inorganic salts. The medium used for raising fast growing friable callus is usually suitable for initiating cell suspension culture. However, manipulation of the auxin or cytokinin ratio could be done to achieve culture with better dispersion of the cells (Bhojwani and Razdan, 1996). The cell suspension culture needs to be agitated on the shaker in order to break up the cell aggregates, maintain the culture with a uniform distribution of cells of various sizes and shapes,

and to provide gas exchange for the cells to sustain cell growth (Jha and Ghosha, 2005).

2.3.4 *In Vitro* Production of Secondary Metabolites

The application of *in vitro* plant culture techniques in the pharmaceutical industry is becoming more important to ensure a sustainable supply of the source materials which is now becoming more difficult due to the factors like environmental changes, diverse geographical distribution and over exploitation by the pharmaceutical industry itself. Plant cell culture which is biosynthetically totipotent, can be an attractive alternative to traditional cultivation method in producing plant derived compounds, especially if the source plant is difficult to cultivate, has long cultivation period or has very low metabolite yield. An excellent example is the Pacific Yew tree, *Taxus brevifolia* which produces the anti cancer drug, Taxol®. This tree, which was originally found in Pacific North-West, is very slow growing. It requires more than 1000 barks of up to 100 years old *Taxus brevifolia* trees to produce 1 kg taxol. Thus, in this case plant cell culture may provide an alternative production mechanism for sufficient supply of the drug and to assure that the species population is conserved (Kieran *et al.*, 1997).

Many attempts have been made to improve the production of secondary metabolite in plant cell cultures. Several interesting strategies were reviewed by Rao and Ravishankar (2002). The first strategy is screening and selection of highly productive cell lines. Screening starts with selecting a parent plant with high contents of the desired products to induce several callus lines. Then, the heterogeneous callus lines are screened to obtain high-producing cell lines using several methods. Ogino *et*