THE ESTABLISHMENT OF EMBRYOGENIC CALLUS CULTURE OF *Hyoscyamus niger* AND THE DETECTION OF HYOSCYAMINE IN THE CULTURE

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by

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LIST OF ABBREVIATIONS

MS	Murashige and Skoog
Kin	Kinetin
NAA	Naphathaleneacetic acid
BA	6-benzylaminopurine
IBA	3-indole butyric acid
TDZ	Thidiazuron
2,4-D	2,4-dichlorophenoxyacetic acid
Picloram	4-amino-3,5,6-trichloropicolinic acid
GC-MS	Gas Chromatography- Mass Spectrometry
TIC	Total Ion Chromatogram
v/v	Volume per volume
ANOVA	Analysis of Varians
TBA	Tertiary butyl alcohol
Dicamba	Dichloro-o-anisic acid

THE ESTABLISHMENT OF EMBRYOGENIC CALLUS CULTURE OF *Hyoscyamus niger* AND THE DETECTION OF HYOSCYAMINE IN THE CULTURE

ABSTRACT

Embryogenic callus of *Hyoscyamus niger* could be induced from the leaf, petiole and root explants however the best embryogenic callus induction was obtained from the leaf explants cultured on MS medium supplemented with 6.0 mg/L picloram after four weeks of culture. The embryogenic callus could undergo maturation stage on MS supplemented with 1.0 mg/L BA followed by one week culture on MS basic medium. The addition of casein hydrolylasate in the MS + 1.0 mg/L BA medium generally induced higher biomass of embryogenic callus and more globular and torpedo shape embryos. However, the mature somatic embryos failed to germinate in the germination medium, MS supplemented with 0 - 10.0 mg/L BA. Embryogenic cells of *H. niger* could be propagated by culturing the friable embroygenic callus in liquid MS medium supplemented with 6.0 mg/L picloram and subculturing was carried out every 12 days. Transferring the embryogenic cells into liquid MS medium only produce root without showing any sign of maturation. Preliminary study showed that direct embryogenesis could be induced from the leaf and petiole explants cultured on MS medium supplemented with BA (2.0 - 8.0 mg/L) while only petiole explants showed direct embryogenesis formation on MS medium supplemented with 6.0 mg/L BA and 0.5 mg/L NAA. Histological study confirmed the formation of both indirect and direct embryogenesis of *H. niger*. GCMS analysis revealed the presence of hyoscyamine in the *H. niger* embryogenic callus induced on MS supplemented with 1.0 mg/L BA after culturing on MS for one week and

embryogenic callus and somatic embryos cultured on MS + 500 mg/L casein hydrolysate.

KEPERHASILAN KULTUR KALUS EMBRIOGENIK *Hyoscyamus niger* DAN PENGESANAN HIOSIAMIN DALAM KULTUR

ABSTRAK

Kalus embriogenik *Hyoscyamus niger* boleh dihasilkan dengan menggunakan eksplan daun, petiol dan akar namun penghasilan kalus embriogenik yang terbaik diperolehi melalui eksplan daun yang dikulturkan dalam medium MS yang ditambah dengan 6.0 mg/L picloram selama empat minggu. Kalus embriogenik yang dihasilkan di dalam medium tersebut menjalani proses pematangan setelah dikulturkan di dalam medium MS selama satu minggu diikuti dengan pengkulturan dalam MS yang ditambahkan dengan 1.0 mg/L BA. Biomass kalus embriogenik dan bilangan embrio globular dan torpedo yang tinggi dapat dihasilkan dengan penambahan kasein hidrolisat di dalam medium MS yang ditambah dengan 1.0 mg/L BA. Namun demikian embrio somatik gagal membentuk plantlet di dalam medium percambahan, MS yang ditambah dengan 0-10.0 mg/L BA. Sel-sel embriogenik *H. niger* boleh dipropagasi di dalam medium MS cecair yang ditambahkan dengan 6.0 mg/L pikloram dan pensubkulturan dilakukan setiap 12 hari. Sel-sel embriogenik yang dipindahkan ke dalam medium cecair MS hanya menghasilkan akar dan tidak menunjukkan tanda pematangan. Kajian awal menunjukkan embriogenesis secara langsung boleh dihasilkan dengan menggunakan eksplan daun dan petiol yang dikulturkan di dalam medium MS yang ditambahkan dengan BA (2.0 – 8.0 mg/L) manakala hanya eksplan petiol dapat menunjukkan penghasilan embriogenesis di dalam medium MS yang ditambahkan dengan 6.0 mg/L BA dan 0.5 mg/L NAA. Pengenalpastian proses embriogenesis secara langsung dan tidak langsung di dalam kajian ini telah disokong melalui kajian histologi. Kajian analisis GCMS yang dijalankan telah dapat menunjukkan kehadiran hiosiamin di dalam kalus embriogenik yang dikulturkan di dalam medium MS yang ditambahkan dengan 6.0 mg/L

pikloram dan kalus embriogenik dan embrio somatik yang dihasilkan di dalam medium MS yang ditambahkan dengan 500 mg/L kasein hidrolisat.

1.0 INTRODUCTION

Since the civilization of man, they have relied on Nature to supply for their basic needs such as the production of food-stuffs, shelters, clothing, means of transportation, fertilizers, flavors, fragrances and medicine. Plants are also used as sources of many compounds and mixtures of compounds to appease the needs of human. These compounds, which are referred to as secondary metabolites, include drugs, flavors, enzymes, essential oils, and colorings. The use of plant-derived secondary metabolites as alternative sources of herbal preparations continues to play a major role in the up keep of general wellness of people all over the world. Several important pharmaceuticals have been discovered from plants and their properties have been investigated for more than 200 years. The chemical diversity of plants is greater than any chemical library made by humans and thus the plant kingdom represents an enormous reservoir of pharmacologically valuable substances waiting to be discovered (Oksman-Caldentey and Inzé, 2004). In modern pharmacy, about 25% of drugs still contain active compounds from natural sources, which are primarily isolated from plants (Oksman-Caldentey and Hiltunen, 1996). According to report from WHO, 80% of the world's population, primarily those of developing countries, rely on plant-derived medicines for their healthcare (Gurib-Fakim, 2006).

Many plants from the Solanaceae family are considered as medicinal plants. The tropane alkaloids, found mainly in the plants of this family, contain the anticholinergic drugs hyoscyamine and scopolamine. Solanaceous plants, used traditionally for their medicinal, hallucinogenic and poisonous properties, were due to these tropane alkaloids (Li *et al*., 2006).

Hyoscyamus is a highly diversified genus in the Solanaceae family and comprises twelve to fifteen species, most of which are native to the Mediterranean region (Heiser, 1969). *Hyoscyamus niger* (black henbane) has been used medicinally for centuries and is commercially cultivated in Europe for its alkaloid compounds. All plant parts contain tropane alkaloids (hyoscyamine, scopolamine, atropine) and are toxic to humans and animals when they are ingested in large amount. *H. niger* has been used for the treatment of motion sickness, asthma and as anaesthetic agent.

Although the dramatic progress of organic chemistry has resulted in an enormous production of synthetic drugs, some of the most powerful remedies are still of plant origin. Organic synthetic of complicated molecules is often extremely costly, plants produce them with apparent ease and at little cost, hence plants will remain as the important source of useful compounds. However, the quantity of plant species available for extraction of a particular secondary metabolites may be limited by numerous factors. These factors include natural restriction of the geographical area in which the plant grows, transportation problems, difficulties of collecting plants of a particular species that grows at low population densities, and quarantine requirements.

Hence, tissue culture has been suggested as an alternative method for the production of these phytochemicals. Application of tissue culture in medicinal plants has proven to be useful in the production of therapeutic compounds. With this vital tool, the desired constituents may be obtained during a short period without the destruction of the entire plant. Some chemical constituents have proven to be extracted via tissue culture techniques. Efforts have been made to develop economically feasible methods for the production of tropane alkaloids *in vitro* culture techniques by many researchers since West and his team found tropane alkaloids in

Atropa belladonna callus more than 30 years ago (Misawa, 1994; Khanam *et al*., 2000; Bensaddek *et al*., 2001; Kang *et al*., 2004; Jordan *et al*., 2006). This technique eliminates the factors of weather, temperature, physical barriers, season, and pests on the growth of cultures which are controlled under standard conditions. It also requires less space. Besides its uses for the rapid vegetative propagation of plants, tissue culture has at least four other applications of commercial potential: the removal of diseases from plants, particularly viruses; conservation of disease-free stocks plants; facilitation of genetic change and genetic engineering of plants and production of valuable chemical products (George and Sherrington, 1984). Somatic embryogenesis is one of the *in vitro* techniques for propagating plants. It is defined as a developmental process of somatic cells that resemble zygotic embryos (Khaleda and Al-Forkan, 2006). The process of somatic embryogenesis is important for the production of plantlets and secondary products.

Hyoscyamus niger is a biennial plant originates from Europe. Being a temperate plant, it is difficult to cultivate the plant in the tropics. To date, the *H. niger* plants were imported by the local pharmaceutical companies from United States, Australia and the United Kingdom for obtaining the tropane alkaloids from the plant extracts. The cost of this imported plant material become the additional cost of production of tropane alkaloids and hence make it very expensive.

Lai (2003) had proven that tropane alkaloids could be obtained via micropropagated plantlets and the root cultures of *H. niger*. This research was undertaken to study the production of embryogenic callus, the other aspect of tissue culture techniques, for the production of tropane alkaloids. Hence, the objectives of this research are :

1. To produce embryogenic callus from different plant parts of *H. niger* and to detect the tropane alkaloids contained in the embryogenic calluses.
2. To establish the best culture medium for the induction of somatic embryos of *H. niger*
3. To develop indirect embryogenesis protocol for the production of the tropane alkaloids from *H. niger*
4. To study the possibility of direct embryogenesis of *H. niger* using vegetative explants.

2.0 LITERATURE REVIEW

2.1 History and distribution of *Hyoscyamus* species

The Solanaceae, commonly known as the nightshade family, consists about 96 genera with 3000 species. They grow widely in South America, widespread in the temperate areas and grow with difficulty in some of the tropical regions. This family consists of a wide variety of economical, agricultural and pharmaceutical importance plants (Wink, 2003). According to Hanus *et al*. (2005) the Solanaceae ranks as one of the most important plant family to human beings and they are extremely diverse. Species of this family are used as food (the potato and the tomato), drugs (tobacco; deadly nightshade; mandrake) and ornamentals (petunia; velvet tongue or butterfly flower). The plants of the solanaceae family are also rich in alkaloids but the tropane alkaloids are found only in 15 genera of this extensive family. Six of these plant genera are commercially important such as *Atropa belladonna* and *Hyoscyamus niger* (Heiser, 1969).

Many plants that contain tropane alkaloid have long been utilized for their medicinal, hallucinogenic and poisonous properties (Dräger, 2006). Historically, the use of alkaloid-containing plants of the solanaceae family can be traced back to the beginning of civilization. It was reported that Cleopatra used the atropine-containing extracts of Egyptian henbane (*Hyoscyamus muticus*) to dilate her pupils and thereby appear more alluring during the last century B.C. Medieval European women utilized extracts of deadly nightshade, *Atropa belladonna*, for the same purpose (Kutchan, 1995). Mandrake (*Mandragoras officinarum)* were used during the Roman times in traditional rituals (Ramaoutsaki *et al*., 2002). Al Qur'an (2005) carried out a study on toxic plant species in the southern part of Jordan and had

classified *Hyoscyamus albus* as a popular toxic plant species that caused gastric inflammation and abdominal disturbances. *H. muticus* or As-sakran can be found in scattered localities in the northern region of Saudi Arabia (Rahman *et al*., 2004)

Hyoscyamus species have their origin in Africa and South west Asia and the best known is the poisonous weed henbane, *Hyoscyamus niger*, a natural source of hyoscyamine, hyoscine and atropine (Dräger, 2002). Other synonyms and common names for *Hyoscyamus niger* are common henbane, devil's eye, foetid nightshade, henbell (Anglo-Saxon), hogs bean, Jupiter's bean, poison tobacco, stinking nightshade, symphonica, cassilato or cassilago (Heiser, 1969). In India, it is also known as parasikaya (Sanskrit), khurasaniayavayan (Hindu), khora-saniajowan (Bengal), khurashanivamam (Telegu) and kurasaniyomam (Tamil). Arabic name for this plant is Bazrul-banj (Khory and Katrak, 1984) and is also known as Damtura in Sind (Murray, 1984). In northwestern Anatolia, Turkey, *H. niger* is known by the locals as diş otu or goz otu (Tuzlaci and Aymaz, 2001) while in central Anatolia, it is known as devdala otu or kumacik otu (Sezik *et al*., 2001).

H. niger is widely distributed in Europe and Asia (Sajeli *et al*., 2006). This plant can be found at slopes, roadsides, sandy bank rivers, and occasionally cultivated in rich humus soils near villages and houses in China, Afghanistan, India, Japan, Korea, South west Asia, North Africa and throughout Europe. According to Deb (1979), *H. niger* is distributed from Tibet to Kashmir and Pakistan, between 1500 m and 2300 m in altitude. The plant grows wild in abundance throughout the Himalayan range at altitudes ranging from 8,000 to 11,000 feet. It has also been successfully cultivated in India at the Botanical Gardens of Saharanpur, Poona and Calcutta (Dey, 1986). Macrofossil seeds of *H. niger* from five settlements sites in southern Finland were recorded and the oldest came from Rapola, Sääksmäki and

dated to the Viking Age (Lempiäinen, 1999). Halpern (2004) reported that *H. niger* was introduced into the United States as ornamental flowers. The plant can also be found in waste lands and disturbed ground in Britain and Europe, mainly on lowlands.

2.2 *Hyoscyamus niger*

2.2.1 Botany of *Hyoscyamus niger*

Hyoscyamus niger grows as an annual or as biennial plant. The former has a long-day flowering habit, while the latter responds to a cold treatment (vernalization), sometimes combined with a spesific photoperiodic regime to initiate flowers (Raghavan, 1986). It grows from one to one-half feet tall and it's leaves are ovate, long and large with greenish grey colour and grandular hairy surface (Khory and Katrak, 1984). The flowers are almost stalkless and the bell-shaped corolla is yellowish with purple veins. The calyx enlarges as the fruit develops and surrounds the many seeded capsule (Heiser, 1969). It produces a huge amount of seeds ranging from 10,000 to half a million seeds per plant, and as few as 10-20 seeds are enough to poison a child (Mitev, 2000). The seeds contain hyoscyamine, a fixed fatty oil, an empyreumatic oil and ash (Khory and Katrak, 1984). The unbranched stem produces unpleasant odour with acrid taste. All parts of the plant are poisonous (Wren, 1956). It is propagated by seeds or cuttings (Prajapahit *et al*., 2003).

2.2.2 Uses of *Hyoscyamus niger*

H. niger have been used for medicinal, religious and ceremonial purposes. All parts of the plant are with medicinal values, but the leaves and seeds are usually employed (Wren, 1956). Husain (1983) reported that the leaves contained 0.01% -

0.1% of the total alkaloids with approximately 75% hyoscyamine and 25% scopolamine (hyoscine). It was used in ancient Greece to evoke prophecies and in the Middle Ages to conjure up demons and to give the gift of prophecy (Keeler and Kane, 1967). *H. niger* was reported to produce a more narcotic effect when mixed with tobacco. During the Middle Ages, the witches used it in their rituals. The Egyptians smoked it to relieve toothache (Heiser, 1969).

Roman and Byzantine scientists, such as Dioscorides Pedanios, Gaius Plinius Secundus, Galen, Orivasios from Pergamum, Themistios, Aetios of Amida, Ioannes Damascenos and others, considered *H. niger* as the most important herb, besides the opium poppy, for relieving pain and providing sedative effect. Pliny and Aeginites physicians during the Roman period (2^{nd} century BC to 4^{th} century AD) reported that the plant not only used as narcotics and analgesic but could also be used for treating other diseases or ailments such as ulcers, ear and eye inflammation, rheumatism, cough due to tuberculosis and fever (Ramoutsaki *et al*., 2002). The oil extracted from the seeds can be used for making soap. In Turkey, the seeds of *H. niger* were used as folk medicine. The seeds were boiled in water and added in steam bath as a treatment for conjunctivitis (Tuzlaci and Aymaz, 2001).

In India, the seeds of *H. niger* made into a paste with mare's milk and tied up in a piece of wild bull's skin, were believed to inhibit conception if worn by women (Deb, 1979). The plant is a milder deliriant compared to *Atropa belladonna* but is more hypnotic and quick acting. It was also used to counteract the griping action of purgatives and to relieve spasms in the urinary tract. Poultices, plasters and medicated oils prepared from the leaves and seeds were used to relieve inflammatory swellings.

In the 13th and 14th centuries, the witches had narcotized themselves by a special salve containing extracts of *Atropa belladonna* and henbane. When it spread onto the skin, it caused vivid hallucinations of flying in the air, wild dancing and abundant feasts (Mitev, 2000). *Hyoscyamus niger*, *Atropa belladonna* (deadly nightshade), *Datura strammonium* (jimson weed) and mandrake root were used for their analgesic effect, in combination with poppy in 2500BC (Yang, 2002)

Nowadays, henbane is cultivated as a rich source of alkaloids for the pharmaceutical industry. The alkaloids from *H. niger* are hyoscyamine and scopolamine which have anticholinergic action (Jung *et al*., 2001). Both are used for dilating pupils for fundoscopy and are used in anesthesia to treat per prevent bradycardia and to decrease bronchial and salivary secretions. Atropine is also one of the medicines used for treating organophosphate poisoning and exposure to nerve agent chemical weapons.

Scopolamine obtain from *H. niger* is used commonly for the treatment of motion sickness. This compound causes toxic delirium when overdose with symptoms such as amnesia, confusion, dissociation, hallucinations, delusions, and an excited, giddy affect. Coordination is also impaired, vision becomes blurry with increasing pupillary dilation, and overly dry mucous membranes may make it difficult to talk or swallow. Overdose can be lethal with fever, tachycardia, and arrhythmia while some symptoms such as headache and mydriasis can persist for weeks (Halpern, 2004; Halpern and Sewell, 2005). Patients were found to demonstrate anticholinergic syndromes after ingesting henbane (Spoerke *et al.,* 1987).

In recommend doses, *H. niger* is used as a mouthwash and painkiller. However, in large doses it may cause delirum, hallucinations and rapid heartbeat. This is because the plant is known to block the effects of parasympathetic nervous system thus causing increased heart rate and dilated pupils. Basically, it will not be any problem if the drug is taken by people under 45 years old for a short period of time. However, women in pregnancy, breastfeeding or planning to get pregnant should not take the drug.

Smoking or eating the leaves results in intoxication, but dangerous overdose is possible from the different plant parts with the seeds being particularly toxic (Halpern, 2004). Numerous cases of intoxication and deaths of animal and humans have been reported following consumption of parts of the plants. It was reported that in Turkey during the year 1982 to 1983 intoxication among children due to the abuse of black henbane plants had resulted two out of 76 children died while five of them in a comatose condition (Tugrul, 1985). Betz *et al.* (1991) reported cases of oral poisonings with *Hyoscyamus niger* and another nightshade plant, *Datura stramomium*. In Switzerland, 152 incidences of plant poisoning were due to black henbane (Jaspersen-Schib *et al.*, 1996).

2.3 Somatic embryogenesis

The term tissue culture is applied to any non differentiated cell culture grown on solid or as suspension culture in liquid medium (George and Sherrington, 1984). The earliest study of plant tissue culture was reported after Haberlandt proposed the culture of single cells using artificial medium in 1902. At that time his experiment was not successful. In 1943 White and team managed to culture plant cells using tomato root explants on solidified agar medium supplemented with inorganic salts,

yeast extract and sucrose. Since then the plant tissue culture techniques were widely used by many researchers for various objectives.

The tissue culture system allows the propagation of selected genotypes with high multiplication rates in an aseptic, temperature-controlled environment. This system also allows the reduction of space requirements and, consequently, labor costs for the maintenance of germplasm collections. Plant materials can be maintained *in vitro* by using slow-growth culture medium for longer duration at normal room temperature or by cryopreservation methods (Vajrabhaya, 1988). Organogenesis and embryogenesis are the two other developmental pathways by which plantlet formation and secondary metabolites production are achieved for the angiosperms and gymnosperms (Raghavan, 1988).

Many somatic plant cells can be regenerated from different plant parts to produce complete plantlets via somatic embryogenesis. This is based on the totipotency principle which means that each cell possesses and can express the total genetic potential to form fully fertile and complete plant body (Berz and Oksman-Caldentey, 2002). Somatic embryogenesis has been under intensive study since the first report of plantlet development in carrot tissue cultures (Steward *et al*., 1958). Until today, several plant species have been produced by somatic embryogenesis such as cassava (Danso and Ford-Lloyd, 2002), orange (Niedz *et al*., 2002), sunflower (Thomas *et al*., 2004), Japanese black pine (Maruyama *et al*., 2005), prickly pear (Gomes *et al*., 2006), North American ginseng (Zhou and Brown, 2006) and many others.

Somatic embryogenesis is a developmental process of somatic cells. It resembles zygotic embryogenesis. It leads to the formation of embryos from somatic (sporophytic) tissues, such as leaves and cotyledons (Kintzios *et al*., 2000). Embryos

that arise from the vegetative cells of the plant are called somatic embryos (Monnier, 1990). Williams and Maheswaran (1986) defined somatic embryogenesis as a process by which haploid or diploid somatic cells develop into differentiated plants through characteristic embryological stages without fusion of gametes. According to Von Arnold *et al*. (2002), somatic embryogenesis is a process in which a bipolar structure, resembling a zygotic embryo, develops from a non-zygotic cell without a vascular connection with the original tissue. It is a multi-step regeneration process starting with formation of proembryogenic masses, followed by somatic embryo formation, maturation, dessication and plant regeneration. Kantharajah and Golegaonkar (2004) defined somatic embryogenesis as the process by which somatic cells developed through the stages of embryogeny until the formation of whole plant.

Theoretically, a culture initiated form a single explant can produce an unlimited number of embryos. Thus *in vitro* somatic embryogenesis potentially offers alternative form of large scale production of plants (Ananthakrishnan *et al*., 1999; Andrade and Merkle, 2005). Park *et al*. (2005) reported an *in vitro* methodology for mass propagation of *Eleutherococcus koreanum* by using bioreactor and able to produce large number of somatic embryos, plantlet and adventitious roots. Mass propagation via somatic embryogenesis using bioreactor also has been established in *Elaeis guineensis* (Gorret *et al*., 2004).

Besides adaptable to large-scale production of plants, somatic embryos can also be used for biotechnological applications such as gene transfer. Transgenic somatic embryos carrying BTVP2 gene comprising neutralizing epitopes for expression in peanut had been obtained to develop subunit vaccine in plant system for efficient control of Bluetongue disease (Athmaram *et al*., 2006). Somatic embryogenesis also provides as a means for rapid clonal propagation of large

quantities of virus free plants. In a study done by D'Onghia *et al*. (2001), somatic embryogenesis was proven to eliminate *Citrus psorosis virus* (CPsV) from three species of citrus. There were no CPsV detected in regenerated plantlets after ELISA testing. Somatic embryogenesis is used as a cloning technique for rapid and exponential multiplication of particular genotypes (plant hybrids or male sterile plants). It is also a suitable method for maintaining genetic variability created by cellular or genetic manipulations. Analysis of 100 somatic embryos derived plants of *Cephalis ipecacuanha* showed that all plants were morphologically normal and possessed the normal diploid chromosome number of 2n = 22 (Rout, 2000).

In somatic embryogenesis, shoot and root meristems necessary for complete plant development are initiated in one step. Thus regeneration of plants through somatic embryogenesis is often advantageous than organogenesis for plant breeding programs. In some cases, plants regenerated from somatic embryogenesis may develop from a single cell and may exhibit less variability. Furthermore, plants regenerated through organogenesis are not appropriate for genetic transformation due to formation of chimeric plants (Khalil *et al*., 2002).

Another advantage of somatic embryogenesis is that it does not require time consuming subculture steps to increase clonal stock and may overcome difficulties with micropropagation of difficult to root species. Akutsu and Sato (2002) proved that regeneration of plants via somatic embryogenesis was better than organogenesis in terms of reduced culture time and able to produce more regenerants. The total time required for regeneration of *Alstroemeria pelegrina* (L.) var. *alba* and *A. magenta* plantlets via somatic embryogenesis was three months compared to organogenesis technique that required approximately eight months to regenerate plantlets.

A common characteristic of embryogenic tissues is that it can remain embryogenic for a long period of time. Embryogenic tissues of *Abies numidica* were capable of continuously forming somatic embryos over a period of three years (Vooková and Kormuťák, 2002) while embryogenic cultures of *Larix decidua* could be maintained up to 17 years (Aderkas *et al*., 2003). Maintenance of an embryogenic state may require exogenous auxin, or in the absence of exogenous plant growth regulators.

As a method, embryogenesis can be automated, thereby enabling massive quantities of seedlings to be produced. The encapsulation of somatic embryos has also been proposed for obtaining artificial seeds. Artificial seeds offer potential for storage and propagation of elite germplasm. In addition, encapsulation provides physical protection to the somatic embryos as well as protection from pathogens by the inclusion of antibiotics and fungicides. Choi and Jeong (2002) had successfully encapsulated the somatic embryos of Siberian ginseng as artificial seeds. More than 100 American chestnut somatic seedlings were successfully acclimatized to green-house conditions by Andrade and Merkle (2005).

There are two different forms of somatic embryogenesis that leads to the regeneration of plantlets. Somatic embryos can be differentiated either directly from the explant without an intervening callus phase or indirectly after a callus phase (Von Arnold *et al*., 2002). Callus are coherent but organized and amorphous tissue, formed by the vigorous division of plant cells. It is often induced in or upon parts of an intact plant by wounding, by the presence of insects or micro-organisms, or as a result of stress (George and Sherrington, 1984**).** Direct embryogenesis occurred at the surface of the explant tissues. Martin (2003) has established direct somatic embryogenesis of *Anacardium occidantale* from seed coat explants while Gogate and

Nadgauda (2003) reported the success of direct somatic embryogenesis of the same plant from immature zygotic embryos. Direct embryogenesis also obtained from different parts of intact seedlings of *Azadirachta* (Gairi and Rashid, 2004). By contrast, in indirect embryogenesis, the explant tissues first proliferated to give rise to embryogenic callus before any morphogenetic processes could be detected (Stefanello *et al*., 2005). Usually embryogenic callus is formed in auxin containing medium. To date, plants that had been regenerated via indirect somatic embryogenesis were *Olea europea* (Shibli *et al*., 2001), *Agapanthus praecox* (Suzuki *et al*., 2002), *Phlox paniculata* (Jain *et al*., 2002) and *Scirpus robustus* (Wang *et al*., 2004).

According to Arnold *et al*. (2002), process of somatic embryogenesis can be divided into different phases, each of which has its own specific hormonal requirements. Plant regeneration through somatic embryogenesis includes the following five steps :

1. Initiation of embryogenic cultures by culturing the primary explant on medium supplemented with plant growth regulators, mainly auxin but often also cytokinin.
2. Proliferation of embryogenic cultures on solidified medium or in liquid medium supplemented with plant growth regulators, similar to initiation medium.
3. Prematuration of somatic embryos in medium lacking plant growth regulator; this inhibit proliferation but stimulates somatic embryos formation and early development.

4. Maturation of somatic embryos by culturing on medium supplemented with ABA and/or reduced osmotic potential.
5. Regeneration of plants on medium lacking plant growth regulator.

Plant regeneration via indirect somatic embryogenesis require the production of embryogenic calluses or embryogenic cell suspension cultures. For large propagation, suspension cultures are usually employed. The proliferation rate is higher and the cultures become more synchronized. In suspension cultures, single cells and cell aggregates develop as separate structures. Thus the cells can easily be separated by sieving or centrifugation, and thereafter, subculture and manipulate as required. There were several reports of production of somatic embryos through cell suspension cultures in various plants: sandalwood (Das *et al*., 2001), carrot (Li and Kurata, 2005), horsegram (Mohamed *et al*., 2005), potato (Vargas *et al*., 2005) and banana (Strosse *et al*., 2006),

Many factors can affect the development of embryos such as the plant material (genotype, source, physiological stage of explant), culture medium (minerals, plant growth regulators, supporting agents) and environment (temperature, illumination properties). Somatic embryogenesis is influenced by explant source and genotype. A wide range of plant tissues has been used as explant source from which to obtain somatic embryos. The choice of explant is considered to be an important factor in the induction of somatic embryos. In some species, embryos may be induced from cotyledons (Ramarosandratanam, 2004), leaves (Te-chato and Rungnoi, 2000**;** Devi *et al*., 2004), seeds (Lee and Lee, 2003), stamens (Stefanello *et al*., 2005), petioles (Zlenko *et al*., 2005) and roots (Chen and Chang, 2000; Ishizaki *et al*., 2002). Plant genotype can be a critical factor for the success of somatic embryogenesis. For example, there were differences in the embryogenic response

from different coffee genotype depending on the month of the year in which the leaf explants were taken, but those differences were not related to climatic elements (Molina *et al*., 2002). Different responses were also observed in the induction of somatic embryos in different genotype of coconut (Fernando and Gamage, 2000). Kumar *et al*. (2003) had proven that two different cultivars of *Cucumis sativus* showed different responses towards the formation of somatic embryos.

Culture medium is another key factor to a successful embryo induction and subsequent plant regeneration. Embryogenesis from the cultured explants is dependent upon an exogenous application of auxin and its concentration. The auxins used for callus initiation are almost exclusively 2,4-dichlorophenoxyacetic acid (2,4-D) or naphathaleneacetic acid (NAA). These auxins are usually used in combination with a cytokinin either 6-benzylaminopurine (BA) or kinetin (KIN). Somatic embryos from various cultivars of Greek squash were obtained by culturing cotyledon and leaf explants on a medium containing 22.6 μM 2,4-D and 15.5 μM KIN (Kintzios *et al*., 2000). Embryogenic callus was induced using 2,4-D alone in *Disentra spectabilis* (Lee and Lee, 2003) and the presence of NAA in the culture medium for *Eucalyptus globules* (Pinto *et al*., 2002). Other auxins, such as dichloro-o-anisic acid (Dicamba) and 4-amino-3,4,6-trichloro picolinic acid (Picloram), have been used to enhance somatic embryogenesis. Addition of 30 μM Dicamba to the culture medium of bahiagrass seed explants had produced 21.4% embryogenic callus (Grando *et al*., 2002). Dineshkumar *et al*. (1995) reported a protocol for somatic embryogenesis in chickpea using picloram. An emerging trend in tissue culture is the utilization of Thidiazuron (TDZ), a non-purine phenylurea derivative, for the induction of somatic embryos such as the formation of somatic embryos of rice (Gairi and Rashid, 2004). The frequency of embryogenesis as well as the average

number of embryos formed were affected by the supplement of TDZ into the culture medium for *Cajanus cajan* (Singh *et al*., 2003). On the other hand, combination of TDZ with NAA effectively increased the frequencies of explants with embryo formation from 71% to 94% in *Epipremnum aureum* (Zhang *et al*., 2005).

During recent years, attention has been given to the effect of micronutrients in the culture medium such as Ag^{+}, Co^{2+}, Ni^{2+}, Zn^{2+} and Cu^{2+} towards embryos formation. It was reported that the optimal concentration of copper sulphate for high number of embryos produced in cassava was 5 μM and this concentration also reduced the maturation time of somatic embryos to 25 days from embryo initiation (Danso and Ford-Lloyd, 2002).

Carbon source was found to be affecting the development of somatic embryos. Effects of several carbohydrates such as glycerol, sorbitol, mannitol, lactose, glucose and galactose were studied at different concentration ranges, on embryogenesis of ovule derived-calli from three species and four varieties of citrus (Kayem and Koc, 2006). The use of maltose instead of sucrose was found to enhance somatic embryo production in *Oncidium* (Jheng *et al*., 2006).

Amino acids, notably proline and its analogs such as alanine, glutamine, lysine, and serine have also been reported to increase the frequency of somatic embryogenesis. The use of glutamine, arginine and proline was found to improve the conversion of somatic embryos conversion into plants for *Cryptomeria japonica* (Igasaki *et al*., 2003). Casein hydrolysate (CH) is an undefined composition of different amino acids. Addition of 200 mg/l casein hydrolysate together with proline in the maturation medium incubated in the dark resulted in the increase of callus proliferation and production of primary somatic embryos in banana (Khalil *et al*., 2002). Organic nitrogen is inexpensive and a useful nitrogen source for plant cells

and it is frequently used to stimulate the growth of cultured plant cells and tissues. Transferring friable loose calli into hormon-free MS medium containing higher amount of potassium nitrate (MS + 1.9 g/l KNO_3) further improved the frequency of embryogenesis (Kumria *et al*., 2003).

The addition of activated charcoal to the medium had proven to be useful in many cultures towards somatic embryogenesis including *Manihot esculenta* (Groll *et al*., 2002), *Hyophorbe lagenicaulis* (Sarasan *et al*., 2002) and *Liquidambar styraciflua* (Merkle *et al*., 1998). Activated charcoal originally added to a culture medium was to darken the medium and simulate conditions similar to soil, but it had also been reported to improve most tissue culture responses including somatic embryogenesis, organogenesis, adventitious shoot production, shoot growth and the rooting of micro-propagated tissues (Van Winkle and Pullman, 2005). Kamo *et al*. (2004) reported that the addition of 0.25% activated charcoal on solidified MS basal medium had increased the formation of cotyledonary-stage embryos of two Rosa hybrida cultivars, Kardinal and Classy.

With the presence of high auxin content in a culture medium, a somatic embryo may give rise to new somatic embryos. Such a process is described as secondary, recurrent or repetitive embryogenesis (Parrot *et al*., 1991). Secondary somatic embryogenesis is the phenomenon whereby new somatic embryos are initiated from somatic embryos (Raemakers *et al*., 1994). It has the potential to produce many plants and once initiated, may continue to produce embryos over a long period of time. Little *et al*. (2000) reported that high level (83.0 or 124.4 μM) of picloram or centrophenoxine supplemented into the solid culture medium could induce highly repetitive globular-stage somatic embryos from mature peanut axes.

2.4 The chemical constituents of *Hyoscyamus niger*

2.4.1 Plant alkaloids

The term 'alkaloid' has been defined as a cyclic organic compound containing nitrogen in a negative oxidation state, which has limited distribution in living organisms. Alkaloids are sparsely distributed in the plant kingdom and are much more specific to defined plant genus and species (Borgaud *et al*., 2001). They are mostly derived from amino acids and can be found in about 20% of plant species (Facchini and St-Pierre, 2005). According to Gurib-Fakim (2006), alkaloids are more commonly found in dicotyledons than monocotyledons. The plant species that are rich with alkaloids include plants in the families of Liliaceae, Amaryllidaceae, Apocynaceae, Berberidaceae, Leguminosae, Papaveraceae, Ranunculaceae, Rubiaceae and Solanaceae.

The role of alkaloids in plants are varied and ranged as end products of metabolism or waste products, storage reservoirs of nitrogen, protective agents for the plant against attack by predators or as a substitute for minerals in plants, such as potassium and calcium. Plants that produce alkaloids have been utilized for their medicinal and toxic properties. The widely used plant-derived alkaloids include analgesics (morphine and codeine), stimulants (caffeine and nicotine), and chemotherapeutics (vincristine, vinblastine and paclitaxel) (Dräger, 2002).

There are approximately 12,000 known alkaloids that can be subdivided into several subclasses: proto-, piperidine-, pyrrolidine-, pyridine-, quinolizidine-, tropane-, pyrrolizidine-, imidazole-, purine-, quinoline-, isoquinoline-, quinazoline-, indole-, terpenoid-, and steroidal-alkaloids (Oksman-Caldentey and Inzé, 2004). Pyrrolidine-alkaloids are a small class of alkaloids which can be further subdivided

into simple pyrrolidines typified by hygrine and stachydrine, and the tropanes such as hyoscyamine and scopolamine, and cocaine (Hughes and Genest, 1973).

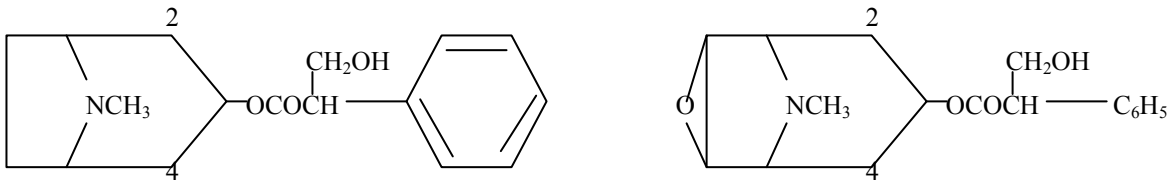

Figure 2.1 Tropane alkaloids (Hughes and Genest, 1973).

Tropane alkaloids (**Fig. 2.1**), with their well-known pharmalogical activities and significant therapeutic importance, are extensively used in modern medicine (Pandey *et al*., 1999; Theodoridis *et al*., 2003; Berkov *et al*., 2005**;** Dräger, 2006). Furthermore, the tropane alkaloids rank among the top ten compounds of plant origin used in pharmaceutical preparations (Rahman and Ahuja, 1998). However, the commercial cultivation of tropane alkaloids producing plants is limited due to their restricted distribution in tropical areas. Many efforts have been made to develop economically feasible methods for the production of tropane alkaloids by applying plant tissue culture techniques (Bensaddek *et al*., 2001**;** Rothe and Dräger, 2002; Kursinszki *et al.,* 2005). Studies on the production of the tropane alkaloids by plant tissue cultures have been ongoing since the discovery of tropanes in *Atropa belladonna* callus tissue (Dicosmo and Misawa, 1995).

2.4.2 The tropane alkaloids of *Hyoscyamus niger*

Hyoscyamine and scopolamine are the two most common alkaloids found in plants of the Solanaceae family (Hashimoto *et al*., 1986; Zehra *et al*., 1998; Palazón *et al*., 2003; Mino *et al*., 2005). The most important tropane alkaloid-producing

plants belong to the genera Datura, Hysocyamus, Atropa, Duboisia and Scopolia (Doerk-Schmitz *et al*., 1994; Asano, 2000; Kang *et al*., 2004; Kitamura *et al*., 2004). These alkaloids possess therapeutic properties and have been used for various medicinal preparations. They are widely used as anticholinergic agents that act on the parasympathetic nerve system (Hashimoto *et al*., 1993).

Hyoscyamine is an alkaloid readily convertible into atropine, which is identical with daturine and duboisine. It is the main constituent of the leaves, juice and seeds of the *Hyoscyamus* spp. and can also be found in plants of *Datura, Duboisia, Atropa* and *Scopolia* genera. Scopolamine represents the main compound of *H. niger* (about 50% of the total alkaloid content) is a sedative and euphoric, in contrary to *l*-hyoscyamine, which has stimulatory and hallucinatory effects (Gaillard and Pepin, 1999). Scopolamine is used against motion disease in the form of an adhesive tape fixed behind the ear from which it is liberated over several hours. Scopolamine as *N*-butyl hydrobromide derivative for oral application acts against spasm of the bladder, the intestine, or the gall bladder. It carries the advantage of having no side effects in the central nervous system due to the quarterny ammonium salt not being transported through the brain blood barrier. Other derivatives of scopolamine such as ipratropiumbromid are anticholinergic drugs inhaled for the treatment of asthma (Halpern, 2004; Dräger, 2006). Because hyoscyamine has undesirable effects on the central nerve system, scopolamine is much preferred in the treatment of gastric disorder and used in the treatment of motion sickness via transdermal patch. Worldwide market for scopolamine is currently estimated about 10 times larger than that of hyoscyamine (and its racemic form, atropine). Thus there has been a long-standing interest in the content of scopolamine in cultivated medicinal plant (Hashimoto *et al*., 1992)**.**

The high demand for these tropane alkaloids from *H. niger* has made it necessary to search for an alternative and biotechnological approach for their production. The synthetic production of this compound is not practically feasible and more expensive than plant extract. Efforts to provide pure compounds of hyoscyamine from *H. niger* were first investigated by Geiger and Hesse in 1833 (Dräger, 2006). According to Miraldi *et al*. (2001), the tropane alkaloids can be obtained from the leaves of *H. niger*. It was mainly synthesized in the roots and transported to aerial parts of the plant where they accumulate in the cell vacuoles at high levels (Hashimoto and Yamada, 2003).

In vitro culture techniques have been proven to be useful tool for production of tropane alkaloid. Detection of the alkaloids in these tissue culture materials through chemical analysis supports the finding. Tropane alkaloids have been obtained from *H. niger* plants with high quantity especially in the roots. However, this plant only grows well in a cool climate and Malaysia's tropical climate is not suitable for large scale planting of this plant. Production of *H. niger* plant materials using *in vitro* culture technique can hence be the alternative mode of production. In this study, somatic embryos production via somatic embryogenesis are being carried out to be used as possible material source for the supply of tropane alkaloids.

The tropane ring system is derived from ornithine and/or arginine by way of putrescine. Biosynthesis of tropane alkaloids involves the formation of pyrrolidine ring that is derived from putrescine via the sequential action of an *S*-adenosyl-*L*-methionine-dependent putrescene-*N*-methyltransferase (PMT), a diamine oxidase and a spontaneous chemical arrangement. *N*-methylputrescene is converted to tropinone, through an incomplete understood pathway that involves the participation

of phenylananine-derived phenyllactic acid. Tropinone is then converted to tropine by tropinone reductase (TRI). Tropine formed by TR-I is esterified with a phenylalanine derivative to give hyoscyamine, which is then converted to the end product scopolamine by hyoscyamine 6-β hydroxylase (H6H; EC1.14.11.11). ψ-Tropine, the product of tropinone reductase II (TR-II), is thought to be converted to calystegines, but little is known about the reactions involved. The metabolic pathway of tropane alkaloids in *H. niger* was proposed as in **Fig. 2.2** (Nakajima and Hashimoto, 1999).