

**MICROPROPAGATION AND CALLUS CULTURE OF
PHYLLANTHUS NIRURI L., *PHYLLANTHUS URINARIA* L. AND
PHYLLANTHUS MYRTIFOLIUS MOON (EUPHORBIACEAE) WITH
THE ESTABLISHMENT OF CELL SUSPENSION CULTURE OF
PHYLLANTHUS NIRURI L.**

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by

ONG POH LIANG

**This is submitted in fulfillment of the requirements
for the “Degree” of: Master of Science**

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*For My Dearest
Family
&
My Best Friends*

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LIST OF ABBREVIATIONS

| | |
|-----------------|---|
| MS | Murashige and Skoog |
| KN | Kinetin |
| IBA | Indole-3-butyric acid |
| Picloram | 4-amino-3,5,6-trichloropyridine-2-carboxylic acid |
| 2, 4-D | 2,4-dichlorophenoxyacetic acid |
| 2-iP | N ⁶ -2-isopentyl-adenine |
| NAA | 1-naphthaleneacetic acid |
| IAA | Indole-3-acetic acid |
| GA ₃ | Gibberelic acid |
| v/v | Volume per volume |
| w/v | Weight per volume |
| PC | Paper chromatography |
| TLC | Thin Layer Chromatography |
| rpm | Rotation per minute |
| PCV | Packed cell volume |
| CRD | Completely Randomised Design |
| CRBD | Completely Randomised Block Design |
| HSD | Tukey' s Studentized Range |
| ANOVA | Analysis of Variance |
| DPPH | 1,1-diphenyl-2picrylhydrazyl |
| DMSO | dimethyl sulfoxide |
| HOAc | Acetic acid |
| BAW | Buthanol: Acetic acid: water in ratio 4:1:5 |

Forestal

Acetic acid: water: conc. HCL in ratio 30:10:3
v/v

SEM

Scanning Electron Microscope

MIKROPROPAGASI DAN PENGKULTURAN KALUS *PHYLLANTHUS NIRURI* L., *PHYLLANTHUS URINARIA* L. DAN *PHYLLANTHUS MYRTIFOLIUS* MOON (EUPHORBIACEAE) DENGAN PEMBANGUNAN PENGKULTURAN AMPAIAN SEL *PHYLLANTHUS NIRURI* L.

ABSTRAK

Satu protocol yang efisien telah dibangunkan untuk menghasilkan anak benih *Phyllanthus niruri* secara besar-besaran melalui proliferasi tunas aksil dengan bahagian nod-nod pokok induknya sebagai eksplan. Bahagian nod-nod pokok yang dikulturkan dalam medium MS yang ditambahkan dengan 1.0 mg/L BA menghasilkan pucuk berbilang maksimum sebanyak 6.6 pucuk per eksplan dalam tempoh empat minggu. Sebanyak 97% daripada pucuk mikro menghasilkan bunga dan buah secara *in vitro* dalam medium MS tanpa sebarang pengawalatur pertumbuhan tumbuhan. Semua pucuk menghasilkan akar dalam medium yang sama. Pembungaan secara *in vitro* pertama diperhatikan selepas 12 hari proliferasi bahagian nod-nod manakala pembuahan berlaku selepas 20 hari pengkulturan. Frekuensi pembungaan dan pembuahan yang paling tinggi (90-100%) diperolehi apabila anak pokok dipindahkan ke medium MS tanpa pengawalatur pertumbuhan tumbuhan selepas kitar pengsubkulturan yang ketiga. Asid giberelik didapati mengurangkan tempoh pembungaan dan merencatkan pembuahan secara *in vitro*. Anak pokok *in vitro* yang ditumbuh baik berbunga dan berbuah dalam medium MS yang ditambahkan dengan 30 g/L sukrosa tanpa sebarang pengawalatur pertumbuhan. Ciri-ciri morfologi buah *in vitro* adalah berbeza daripada buah pokok induknya apabila diperhatikan di bawah mikroskop electron pensakanan (SEM). Permukaan ovari daripada pokok induk adalah kasar dan diselaputi dengan lapisan lilin epikutikular. Manakala permukaan ovary daripada bunga *in vitro* adalah licin tanpa sebarang

pembentukan lilin. Protokol mikropropagasi yang dikembangkan didapati boleh diaplikasikan ke atas *P. urinaria* untuk menghasilkan stok pokok-pokok yang seragam. Kalus rapuh telah berjaya dihasilkan daripada eksplan daun *P. niruri* yang dikulturkan dalam medium MS + 2.0 mg/L picloram (4-amino-3, 5, 6-trichloropicolinic acid). Kalus yang kompak dihasilkan dengan menggunakan medium yang sama daripada *P. urinaria* dan *P. myrtifolius*. Kalus rapuh digunakan sebagai bahan permulaan untuk penyediaan pengkulturan ampaiian sel *P. niruri*. Kinetik pertumbuhan ampaiian sel *P. niruri* adalah bersifat sigmoid dan memasuki fasa pegun pada hari ke-dua belas. MS + 2.0 mg/L picloram dipilih sebagai medium terbaik untuk mengekalkan kultur ampaiian sel. Amaun biojisim ampaiian sel yang tinggi (berat basah dan kering) diperolehi apabila dieramkan dalam keadaan bercahaya dengan intensiti cahaya sebanyak $44 \pm 9 \mu\text{E}/\text{m}^2\text{s}^1$. Aktiviti antioksidan daripada ampaiian sel *P. niruri* menunjukkan kapasiti yang tinggi untuk detoksifikasikan radikal oksigen dengan 1.3- dan 2.0- kali ganda lebih daripada aktiviti antioksidan bagi kultur kalus dan pokok induk masing-masing. Aktiviti antioksidan daripada *P. niruri* adalah berkait rapat dengan kadungan komponen fenolik dalam tisu-tisu tumbuhan. Jumlah komponen fenolik yang paling tinggi telah diperhatikan di dalam kultur sel jika berbanding dengan pokok induk dan kultur kalusnya. Sebanyak 193.2 mg sebatian fenolik (asid galik) terkandung di dalam 1 g ekstrak sel. Jumlah sebatian fenolik pokok induk adalah 138.8 mg/g manakala kultur kalus cuma menghasilkan 43.7 mg/g sebatian fenolik. Nilai R_f bagi tompok kuning terdapat di kromatogram adalah sama dengan nilai R_f kaempferol piawai, maka sel-sel *P. niruri* kemungkinan mengandungi kaempferol.

MICROPROPAGATION AND CALLUS CULTURE OF *PHYLLANTHUS NIRURI* L., *PHYLLANTHUS URINARIA* L. AND *PHYLLANTHUS MYRTIFOLIUS* MOON (EUPHORBIACEAE) WITH THE ESTABLISHMENT OF CELL SUSPENSION CULTURE OF *PHYLLANTHUS NIRURI* L.

ABSTRACT

An efficient protocol was developed for a rapid and large-scale production of the *Phyllanthus niruri* plantlets (Euphorbiaceae) via axillary bud proliferation using nodal segments of the mature plants as explants. The nodal segments cultured on MS medium supplemented with 1.0 mg/L BA produced maximum shoot multiplication with the formation of 6.6 shoots per explants within four weeks. *In vitro* flowering and fruiting occurred in 97% of the microshoots on MS medium without any plant growth regulator. With the same medium, all the shoots produced roots. The first *in vitro* flowering was observed 12 days after initial proliferation of nodal segments while fruiting occurred 20 days after culture. The highest frequency of flowering and fruiting (90-100%) were obtained when the plantlets were transferred to a growth regulator-free MS medium after the third subculture cycle. Gibberellic acid was found to shorten the period of *in vitro* flowering and inhibited *in vitro* fruiting. Complete well growth plantlets with *in vitro* flowers and fruits were observed on MS medium supplemented with 30g/L of sucrose without any plant growth regulator. The morphological features of the *in vitro* fruit was different from the fruit of the mother plant when they were observed under Scanning Electron Microscope. The surface of the ovary from mother plant was rough and coated with a layer of epicuticular wax. While, the ovary surface of the *in vitro* flower was smooth without any superficial wax layer. The established micropropagation protocol could be applied to *P. urinaria* for raising a stock of uniform plantlet but not to *P. myrtifolius*. Friable callus of *P. niruri* was successfully induced from the leaf explants cultured on MS + 2.0 mg/L picloram

(4-amino-3, 5, 6- trichloropicolinic acid). Compact calli were induced by using the same medium for callus induction of *P. urinaria* and *P. myrtifolius*. The friable calli were used as the initiating material for the preparation of the cell suspension culture of *P. niruri*. The growth kinetic of the cell suspension culture of *P. niruri* was characterized by its 'sigmoidal' nature and entered into stationary phase on the twelve day. MS + 2.0 mg/L picloram was the best medium for maintaining cell suspension culture. Higher amount of cell biomass (fresh and dried weight) was obtained when they were incubated under cool white fluorescent lights with light intensity of $44 \pm 9 \mu\text{E}/\text{m}^2\text{s}^1$. Antioxidant activities of *P. niruri* cell suspension cultures were found to possess a higher capacity to detoxify oxygen radicals with a 1.3- and 2.0- fold increase over the antioxidant activity of callus cultures and mother plant respectively. Antioxidant activity of *P. niruri* was associated with the content of phenolic compounds in the plant tissues. The highest total phenolic compound was found in cell culture as compared to the mother plant and callus culture. There was 193.2 mg phenolic compound (gallic acids) contained in 1 g of cell extract. The total phenolic compound of the mother plant was 138.8 mg/g while that of the callus culture was only 43.7 mg/g. The R_f value of the yellow spot found on chromatogram was similar to the R_f value of the standard kaempferol, hence the *P. niruri* cells most probably containing kaempferol.

CHAPTER 1.0 INTRODUCTION

Plants have been used in the preparation of traditional medicine for a long time and most of these folk medicines were prepared from locally grown wild plants. Knowledge about the uses of plants was compiled by trial and error and passed down from one generation to another orally. Nowadays, world markets are turning to plants as the sources of ingredients in healthcare products. Consumers are also more preferred to use plants as producers of secondary metabolites (Holm and Hiltunen, 2002). Plant secondary metabolites were found to be sources of various phytochemicals that could be used directly or as intermediates for the production of pharmaceuticals, as additives in cosmetic, food or drink supplements (Ramlan and Mohamad, 2000).

In recent years, there has been a resurgence of interest in the discovery of new compounds from plants with the aim of finding novel treatment against a variety of illnesses. Many medicinal plants that reported to have the potential for medicinal propose were investigated for useful active compounds. For example *Artemisia annua* L, a medicinal plant traditionally used by the Chinese for fevers and malaria, had resulted to the isolation of artemisinin (qinghao) (Christen and Veuthey, 2001; Charles and Simon, 1990). Garlic (*Allium satrum* L), a medicinal plant since ancient times, was found to have anti-bacterial and anti-fungal activity with the discovery of active compound called allin. It had also been proven to have cholesterol-lowering and anti-hypertensive properties (Khory, 1984).

Phyllanthus niruri Linn is one of the valuable medicinal plants and it has been used for the treatment of various ailments such as flu, dropsy, diabetes and jaundice (Unander and Blumberg, 1991). Interest in this plant was further enhanced due to reports of its anti-tumor and anti-carcinogenic activities and its potential as a remedy for hepatitis B viral infection (Rajeshkumar *et al.*, 2002). It was also found to have high anti-oxidant and hepatoprotective properties (Harish and Shivanandappa, 2006; Chatterjee *et al.*, 2006). Some flavonoids obtained from this plant were reported to have antinociceptive properties (Santos *et al.*, 2000). De Souza *et al.* (2002) reported that the leave of *P. niruri* contained higher amount of phenolic compounds than the branches. While Ishimaru *et al.* (1992) identified six phenolic compounds from this plant which were gallic acid, epicatechin, galocatechin, epigallocatechin, epicatechin 3-o-gallate and epigallocatechin 3-o-gallate. Although the anti-hepatotoxic potential of the plant was controversial, the major components that were responsible for this property were phyllanthin and hypophyllanthin. Niruriside, a new HIV REV/RRE Binding inhibitor was isolated from *P. niruri* using bioassay-guided fractination (Qian-Cutrone *et al.*, 1996).

Various *Phyllanthus* species were found to have various of properties. For instance, *P. urinaria* was used in folk medicine for treating intestinal infections, diabetes, hepatitis B viral infection and disorders of the kidney and urinary bladder (Unander *et al.*, 1995). Several compounds were also isolated from *P. urinaria* such as, rutin, β -amyrin, ellagic acid, gerariin, quercetin and β -sitosterol and reported to have pharmacological effects (Calixto *et al.*, 1998). Besides, six lignan were isolated from *P. myrtifolius*: phyllamycin A, phyllamycin

B, phyllamycin C, retrojusticidin B, justicidin A, and justicidin B. Two of the compounds - phyllamycin B and retrojusticidin B - inhibited HIV-1 reverse transcriptase (RT) at concentrations far lower than those that inhibited human DNA polymerase alpha (HDNAP-(alpha) (Chang *et al.*, 1995).

Many secondary products produced from medicinal plants have been commercialized. The key issue for commercialization of herbal-based products is standardization and consistency of material. Adulteration or even microbial and heavy metal contamination is a potential risk. However recent successes of plant-derived products, increasing in production of the Chinese and Indian herbal medicines, and favorable regulation for commercialization have created a fast growing market for herbal based products and nutraceuticals. The increasing demand for medicinal plants will definitely reduce the sustainable supply of medicinal plants in the future. Moreover, plant secondary products are often produced only in small quantities in most of the plant species. It is not always feasible to isolate secondary compounds from intact plants. Besides, plants are endangered by a combination of factors such as over-collecting, unsustainable agriculture practices, urbanization, pollution and climate change, no proper regulation on management and conservation. Therefore, plant cell and tissue culture techniques can be an alternative approach to maintain sustainability supply of plant materials for producing bioactive compounds continuously under artificially controlled conditions (Thorpe, 2006; Mohd, 2000).

The widespread use of plant *in vitro* culture techniques has many advantages when classical methods of *in vivo* vegetative propagation prove

inadequate. *In vitro* cloning has been proven to be an important tool in speeding up propagation. *In vitro* propagated plants are often healthier than those clone *in vivo*. This is mainly due to rejuvenation and they are often disease-free plants. Cell suspension cultures have also been proven to be suitable for continuous production of biochemicals. The cultured cells are also the material choice for biochemical and molecular investigation of plant secondary metabolites. And scaling up from flasks to bioreactor for the production of phytochemicals is always performed using suspension culture (George and Sherrington, 1984; Pierik, 1997).

P. niruri are grown as weeds in agricultural and waste lands. Most of the people collect this plant from any place without considering whether they are grown in polluted or unpolluted areas. The plants that were collected from polluted site were found to contain heavy metal or toxic components such as mercury (Rai *et al.*, 2005). As these plants are often utilized by human and in order to maintain sustainable supply of healthy and quality plants for human consumption, *in vitro* propagation technique should be used and this has lead to the present study with the following objectives:-

1. To establish the optimum condition for producing *P. niruri* plantlets via *in vitro* culture technique.
2. To produce callus from different plant parts of *P. niruri*.
3. To apply the same proliferation medium of multiple shoots formation and callus induction from *P. niruri* to *P. urinaria* and *P. myrtifolius*.
4. To establish a cell suspension culture system for *P. niruri*.

5. To investigate Dpph free radical anti-scavenging activity and total phenolic compounds from mother plant, *in vitro* plantlet, callus and cell suspension culture of *P. niruri*.

Through this research, it is hoped that the *in vitro* plantlets, callus cultures and cell suspension cultures of *P. niruri* can be used as the material source for the production of useful phytochemical compounds.

CHAPTER 2.0 LITERATURE REVIEW

2.1 Distribution and Uses of *Phyllanthus* spp.

Phyllanthus is a member of the Euphorbiaceae family which comprises over 700 species and has a pan-tropical distribution. *Phyllanthus* genus consisted of about 100 species that were native to Africa and approximately 200 species belong to the new world. Most of the new world species are found in the West Indian region and southern Brazil. There are about 100 species of *Phyllanthus* found in Malaysia (de Padua *et al.*, 1999). In the major modern revision, there are commonly 7 species of *Phyllanthus* found in Malaysia classified as herbs. They are *P. niruri*, *P. urinaria*, *P. deblis*, *P. acidus*, *P. emblica*, *P. pulcher* and *P. reticulatus* (Ridley, 1967).

Many species of *Phyllanthus* are used widely as traditional remedy in South-East Asia, the Pacific, Africa, the Caribbean and South America. *Phyllanthus* plants have been proven to have aphrodisiac, diuretic and purgative properties. They have also been used in the treatment of chest disorders, conjunctivitis, cough, diabetes, diarrhoea, oedema, fevers, hepatitis, nephritis, ophthalmic diseases, small pox and venereal diseases. Some of the *Phyllanthus* species such as *P. emblica* and *P. reticulatus* are used for dye and tanning purposes. Fruits of *P. acidus* and *P. emblica* are eaten as desert while *P. pulcher* is planted as ornamental. The roots of *P. acidus*, *P. emblica* and *P. reticulatus* are used for making utensils and firewoods. Some of the *Phyllanthus* species have shown antibacterial and antifungal activities (de Padua *et al.*, 1999; Unander *et al.*, 1995; Van Holthoon, 1999; Wang, 2000).

2.1.1 *Phyllanthus niruri* Linn.

2.1.1.1 The Biology of *Phyllanthus niruri* L.

The synonyms of *P. niruri* was *P. amarus* Schum & Thonn. and *P. fraternus* Webster. *P. amarus* was probably native to America. It is now found worldwide throughout the humid tropical countries. *Phyllanthus fraternus*, native to northern India and Pakistan, is also occasionally found in America (Unander *et al.*, 1995; Sharma *et al.*, 2005). It is also known as meniran by the Javanese, memeniran by the Sundanese. In Malaysia, it is known as dukung anak, dukong-dukong anak or ramamibuah. The people of Papua New Guinea named it as Manjinimbi while the Philippines called it as kuru kalanggai, or sampasampalukan in Tagalog language. In Thailand, it is known as duuk taitai, makhaam or yaa taibai and Bhumyamalaki. It is called as *Chanca piedra* in Spanish and quebra pedra in Brazil (Paranjpe, 2001; Masturah *et al.*, 2006).

P. niruri is a monoecious annual herb that can grow to a height of 60 cm with phyllanthoid branching of 4 - 12 cm long with about 15 - 30 leaves. The leaves are sessile and elliptical-oblong (5 -11 mm × 3 - 6 mm), obtuse to round at the base, obtuse or rounded and often apiculate at apex. The stipules are ovate-lanceolate to lanceolate. The proximal deciduous branchlets always covered with cymules of one to two male flowers and succeeding axils consists of one male and one female flower. The flowers are pale green with 5 to 6 calyx lobes with scarious margins. The male flower consists of five disc segmented stamens that make up of two to three filaments with free anthers that dehiscing obliquely to horizontally. The female flower was pale green with 5 petals with scarious margins. The flowers were shortly pedicellate with 5-lobed styles free

lokul, appressed to the ovary and bifid in the middle. The fruits, an oblate capsule with 1.0 - 2.5 mm diameter, are obtusely trigonous smooth and the seeds are longitudinally ribbed on the back (Hsuan, 1969).

P. niruri occurs as a weed in open ground, waste land, grossy scrub and dry deciduous forest and usually grows well on humid, sandy soils, up to 1000 m altitude (Heyne, 1987). One of the characteristics of this genus is the phyllanthoid branches which superficially appear to be compound leaves, but the presence of flowers and fruit of the base of each leaf indicates true branches (Unander and Blumberg, 1991).

2.1.1.2 Medicinal Uses of *Phyllanthus niruri* L.

P. niruri has been used in India for about 2000 years in the Ayuverda, Unani and Siddha system of medicine, especially for the treatment of jaundice. From Hainan to Indonesia, a decoction or tea of *P. niruri* is drunk as a diuretic to treat kidney and liver problem, colic and venereal diseases. It is also documented for expectorant (children's coughs), febrifuge, emmenagogue and anti-diarrhetic properties. The pounded plants are applied externally for contusions and skin complaints. A decoction of the whole plant is used as a stomach tonic (Unander *et al.*, 1995 (b); Paranjpe, 2001; Sharma *et al.*, 2005).

In Papua New Guinea, a cooled poultice of the whole plant is used to treat headache or migraine. In India, leaves and fruits of *P. niruri* are grounded into a paste with buttermilk, garlic and peppers, and ingested orally for seven days to treat jaundice. According to Ayurvedic medicine, *P. niruri* has astringent,

deobstruent and antiseptic properties, and is used to treat dyspepsia, dysentery, dropsy, diseases of the urogenital system, gonorrhoea and diabetes. It is applied as a poultice with rice water on oedematous swellings and ulcers. South-American uses *P. niruri* for the treatment of malaria, kidney and bladder stones and urinary disorders. However, the plant has also been reported to induce abortion. Traditional healers from North-eastern Tanzania have used an aqueous extract of the aerial part of the plant for the treatment of diabetes melitus that is not insulin-dependent. In Nigeria, an aqueous extract of dried plants material is used to treat diarrhoea. Leaves are chewed for relieving persistent coughs and stomachache (Sharma *et al.*, 2005; Burkill, 1966). It has been proven to be very useful in folk medicine for the treatment of numerous disorders such as bladder stones and diabetes (Calixto *et al.*, 1984; Unander *et al.*, 1995 (a); Nishiura *et al.*, 2004). It is also employed as antispasmodic, laxative, diuretic, carminative, prevent constipation, fever and other diseases such as malaria, hepatitis B, dysentery, gonorrhoea, syphilis, tubercularis, cough, diarrhoea and vaginitis (Olive-Bever, 1986; Khory, 1984). Apart from these medicinal uses, it was found to have anti-viral property especially against hepatitis virus (Kumaran & Karunakaran, 2007; Oliver-Bever, 1986; Bhattacharjee & Sil, 2006).

P. niruri extract was reported to inhibit the growth of hepatocellular carcinoma induced by NDEA (N-nitrosodietillamin) in animals (Joy and Kuttan, 1998; Rajeshkumar and Kuttan, 2000) and chemically induced liver tissue (Prakas *et al.*, 1995). The anti-tumor and anti-carcinogenic properties of this plant were also proven by Rajeshkumar and Kuttan (2000) and Sripanidkulchai

et al. (2002). Rajeshkumar and Kuttan (2000) reported that it could reduce the toxic side effects of cyclophosphamide and did not interfere with the anti-tumor efficiency of cyclophosphamide. Infusions prepared from the young shoots of *P. niruri* was found to lessen the oedematous swelling and healed ulcers (Mhaskar *et al.*, 2000). The ethanol and dichloromethane extracts of *P. niruri* were reported to inhibit the growth of plasmodium which caused inflammation of gastric intestinal tract (Tona *et al.*, 1999; 2004). Inhibition of HIV was demonstrated by *P. niruri* extract (Qian-Cutrone *et al.*, 1996; Ogata *et al.*, 1992) and was confirmed to be effective for drug resistant HIV strain (Notka *et al.*, 2004). The overall inhibition was due to the antiviral activity targeting at different part of the virus life cycle. Raphael and Kuttan (2003) reported that the *P. niruri* could also cure gastric lesions and possessed anti-inflammation activities.

2.1.2 *Phyllanthus urinaria* Linn. and *Phyllanthus myrtifolius* Moon

2.1.2.1 The Biology and medicinal uses of *P. urinaria* L.

Phyllanthus urinaria has the same morphological and physiological characteristic as *P. niruri*. It is native to Asian tropics and was introduced into America and Africa. Nowadays, it is a pantropical weeds throughout the Malasian region (Ridley, 1967). It has long been used in folk medicine for liver protection, diabetes, hepatitis, jaundice and dropsy (Satyan *et al.*, 1995). A few lignans isolated from *Phyllanthus* plants have been proven to possess cytotoxic and biological activities (Prakas *et al.*, 1995). Huang *et al.* (2002) reported the apoptosis and Bcl-2 down regulation in Lewis lung carcinoma cell triggered by water extract of *P. urinaria*. This has made it to become a potential source to develop a medicine to cure lung cancer.

2.1.2.2 The Biology and Medicinal Uses of *P. myrtifolius* Moon

Phyllanthus myrtifolius has small leaves, 1/4 inch by 3/4 inch long, with short petioles. Leaves line the long arching stems and are arranged on the stem in a flat plane. The small leaves and fine twigs allow it to be transformed into a terrific small-sized bonsai. Even young plants have a rough bark that is very attractive. Additionally, the plant is quick growing and tolerant of varying cultural conditions. Six lignan were isolated from *P. myrtifolius*: phyllamycin A, phyllamycin B, phyllamycin C, retrojusticidin B, justicidin A, and justicidin B. Two of the compounds - phyllamycin B and retrojusticidin B - inhibited HIV-1 reverse transcriptase (RT) at concentrations far lower than those that inhibited human DNA polymerase alpha (HDNAP - α) (Chang *et al.*, 1995). In addition, seven ellagitannins were isolated from *P. myrtifolius* and found to inhibit polymerase DNA activity Epstein-Barr virus at micromolar stage (Liu *et al.*, 1999).

2.2 *In Vitro* Culture Techniques

2.2.1 Micropropagation Technology

Micropropagation technology is being widely utilized commercially in the ornamentals industry and in other plant production organization. This propagation method was widely used after the discovery of plant growth regulators, auxins and cytokinins. The discovery of auxin (IAA) and cytokinin (kinetin) created the great opportunities for *in vitro* propagation of higher plants (Pierik, 1997).

Some *Phyllanthus* spp had been reported to be propagated by *in vitro* culture techniques. Rajasubramaniam and Saradhi (1997) had successfully

induced 14 – 16 shoots from each shoot tip for *P. fraternus* by using B₅ medium supplemented with 10⁻⁵ M BAP. Catapan *et al.* (2000) reported that an average of 21 - 23 shoots could be induced from each nodal segment of *P. caroliniensis* using MS medium supplemented with either 5.0 μM BA, 1.25 – 5.0 μM kinetin or 2.5 – 5.0 μM 2iP. However, 16 - 20 shoots were formed from each nodal segment of *P. urinaria* with the presence of 1.0 mg/L kinetin in B₅ medium. Lee and Chan (2004) reported that multiple shoots could be produced from the nodal segments of *Orthosiphon Staminous* using MS + 0.5 mg/L BA.

Most of the plant cultures could be subcultured once they were established. In fact, subculturing often becomes imperative to maintain the culture or to increase its volume (George and Sherrington, 1984). For example, repeated subculturing of the *in vitro* individual shoot of *Spilanthes acmella* in the proliferation medium could increase the formation of multiple shoot by three folds (Ang and Chan, 2003).

There are five basic stages for successful micropropagation of plantlets. The first stage, the preparative stage or stated as phase zero, involved the correct pretreatment of the starting plant material so as to ensure they are disease free as far as possible. The second phase is the establishment of clean starting tissue for aseptic growth and development. It involves a sterilization protocol for producing aseptic tissues. These aseptic tissues will be used for the next stage of shoot multiplication which can be carried out in a number of ways. Generally plant growth regulators are used for shoot multiplication. The shoots obtained in phase two will be used for root induction at the third phase either *in*

vitro or *in vivo*. Finally, at phase four, the *in vitro* plantlets are acclimatized for better survival when transferred to greenhouse conditions or to the soil (Pierik, 1997).

2.2.1.1 Establishment of Aseptic Explants

The plant tissues or explants collected from the wild or the green house are usually contaminated with microorganisms and other contaminants. These microorganisms such as bacteria or virus must be removed during the preparation of aseptic explants otherwise they would kill the explants either due to their overgrowth or due to the release of toxic substances into the medium. The potential sources of contamination in the cultures are the plant tissues, instruments, culture medium, environment of the transfer area, technicians and incubation room (Dodds and Roberts, 1995).

In fact, the sterilization treatment may vary from season to season as the microbial populations are dependent on seasons (George and Sherrington, 1984). The most commonly used sterilizing agents for obtaining aseptic tissues are sodium hypochlorite and calcium hypochlorite. Sodium hypochlorite is available as a commercial bleach, Clorox[®]. For example, to obtain aseptic apical and axillary buds of *Crossandra infundibuliformis* (L.) Nees, they were surface sterilized with 0.5 % (w/v) Sodium hypochlorite (NaOCl) for 15 - 20 minutes (Girija *et al.*, 1999). Al-Wasel (2000) used 20 % (w/v) Sodium hypochlorite for *Acacia seyal* Del .seeds sterilization. Mercury chloride (HgCl₂) can also be used as disinfectant agent instead of Sodium hypochlorite. Ang and Chan (2003) had proven that 0.08 % (w/v) mercury chloride could be used to obtain aseptic

Spilanthes acmella nodal segments efficiently and 0.1 % (w/v) mercury chloride was used by Tiwari *et al.* (2000) for *Centella asiatica* axillary buds sterilization. Shoot tips of *Catharanthus carandas* cv. Pant Sudarshan, were surface sterilized with HgCl₂ (0.5 %) and NaOCl (1 %) for different durations to obtain shoot explants (Rai and Misra, 2005).

2.2.1.2 Plant Growth Regulators

The most usual groups of plant growth regulators (PGR) used in tissue culture research are the auxins and cytokinins. The amount of PGR in the culture medium was critical in controlling the growth and morphogenesis of the plant tissues (Skoog and Miller, 1957). Generally a high concentration of auxin and a low concentration of cytokinin supplemented into in the medium could promote cell proliferation with the formation of callus. On the other hand, low auxin and high cytokinin concentration in the medium resulted in the induction of shoot morphogenesis. Auxin alone or with the presence of a very low concentration of cytokinin was important in the induction of root primordia (Pierik, 1997).

There are a number of naturally occurring auxins, however, most of these are not generally available for routine use. Because of their stability, synthetic auxins are extensively employed. The most commonly used are 2,4-dichlorophenopxyacetic acid (2,4-D), 1-naphthaleneacetic acid (NAA) and indole-3-butyric acid (IBA). In some chemical compounds which are not strictly auxins, such as dicamba (3,6-dichloro-o-anisic acid) or picloram (4-amino-3,5,6-trichloropyridine-2-carboxylic acid), have been used as auxin to substitute IBA.

Both of these compounds are herbicides when used at higher concentration (Davies, 1987). They are found to occur naturally in many plants including olive and tobacco (Epstein *et al.*, 1989). In many instances, addition of any one of these auxins to a basal medium may be enough to initiate and sustain callus growth. However since there may be different sites of action or target molecules, it can be helpful to use more than one auxin simultaneously or achieving the correct balance of the auxin and cytokinin especially when the tissue is recalcitrant (George and Sherrington, 1984). According to Murthy *et al.* (1998), recalcitrance could be mitigated by the application of other potent synthetic plant growth regulators such as thidiazuron (N-phenyl-n-1,2,3,-thidiazol-5-ylurea).

Tissue culture of monocotyledons, particularly cereal grains and palms, had been achieved in some cases through the use of rather high levels of synthetic auxins like 2,4-D. High levels of auxin could act as herbicides but cell proliferation in the absence of exogenous cytokinin was frequently achieved, Morphogenesis such as the formation of somatic embryos or adventitious organs from callus tissues was observed when the auxin was removed or lowered in the culture medium (Krikorian *et al.*, 1987).

Cytokinins of adenine derivatives are characterized by the ability to induce cell division in tissue cultures usually in the presence of auxin. The most common type of cytokinin found in plants is zeatin. Cytokinin also occurs as ribosides and ribotides. In tissue culture and crown gall culture, cytokinins promote shoot initiation. Lee and Chan (2004) reported that multiple shoots

could be produced from the nodal segments of *Orthosiphon Staminous* using MS + 0.5 mg/L BA. In moss, cytokinins induce bud formation. Kinetin, the prototype molecule for the synthetic adenylyl cytokinins and zeatin which is about 10 times more potent and generally considered the prototype of the naturally occurring cytokinins, are widely used in tissue culture. Dihydrozeatin, also naturally occurring, is not widely used compared to kinetin or zeatin (N⁶-triangle2-isopentenyl adenine) (Davies, 1987). Benson (2000) reported that TDZ (1-phenyl-3-(1,2,3-thia-diazol-5-yl)urea) could display both auxin- and cytokinin-type activities and this was most likely due to it having both phenyl and thiazol groups. Adenine was occasionally added to tissue culture media and acted as a weak cytokinin by promoting shoot formation (Beyl, 2002).

Gibberelic acid (GA₃), the end-product of GA metabolism in *G. fujikuroi*, has been commercially available for many years. Its application to dwarf or rosette plants, dormant buds, or dormant seeds can result in dramatic and diverse effects on growth. GA₃ can also stimulate the production of numerous enzymes notably alpha-amylase in germinating cereal grains. For fruit setting and growth, this can be induced by exogenous applications in some fruit (e.g., grapes). GA₃ can also induce maleness of dioecious flowers (Metzger, 1987). In tissue culture, GA₃ was used for inflorescence proliferation to bypass juvenility and maintain the adult phase as most of the perennial plants usually passed through a long juvenile phase of vegetative development before flowering. Lin *et al.* (2004) reported that ginseng buds were cultured on B₅ medium supplemented with 1 mg/L BA and 1 mg/L gibberellic acid to develop new inflorescences for somatic embryogenesis. The regenerated plantlets from the

embryogenic callus was found to have a juvenile phase and grew normally. Ohlsson & Berglund, (2001) found that giberellic acid could enhance anthocyanin content in the cell culture of periwinkle. This indicated that GA₃ could also enhance the metabolic activity within pathway that lead to stress related secondary metabolites and anthocyanin biosynthesis.

2.2.2 Callus Culture

Theoretically all living cells are capable of giving rise to full plants and this phenomenon is called cellular totipotency. In cultures, isolated plant cells/tissues may be induced to form an actively growing mass of cells called callus which can be multiplied for an indefinite period by routine subculturing. It is an actively dividing and more or less undifferentiated tissue. It can be obtained from isolating tissues, organs and embryos *in vitro*; generally first undergo dedifferentiation before all division starts. It can be considered as a wound response from almost any part of the original plant, both from plant organs (e.g. roots, leaves, petioles and stems) and from specific tissue types or cells (e.g. pollen, endosperm, mesophyll) (Collin and Edwards, 1998). This wound response is characterized by limited cell division and a rapid increase in metabolic activity, but does not necessary lead to callus development. On the other hand, the growth response, resulted in continued cell division and is usually dependent on an exogenous supply of auxin (Allan, 1991). Gautheret and Nobecourt were the first to induce callus culture from carrot (*Daucus carota* L.) root tissues with the aid of IAA (indole-3-acetic acid) in 1939 (Smith, 1988). A portion of the callus tissue when transferred to the differentiation medium could result in shoot or bud regeneration or the formation of somatic embryos (Pierik, 1997).

The age and physiological state of the mother plant could affect the formation of callus. Generally, the explant material should be healthy and vigorous growing. Allan (1991) reported that tissues from plants that were about to enter dormancy were best for callus induction. The importance of plant age was obviously observed from tree species, where callus usually could only be initiated from juvenile tissue, and not explants from mature trees. Matkowski (2004) reported that callus derived from stem and petiole segments collected from the adult plants grew very slowly, became necrotic and eventually died while satisfactory growth took place in root, stem and leaf callus derived from *in vitro* germinated young plants of *Pueraria loata* (Wild.). However, Sahoo *et al.* (1997) reported that callus could successfully be induced from the internode segments of five years old *Morus indica* L. The success in initiation of callus cultures could also depend on whether the mother plant was grown in greenhouse or field grown. The season of the year to collect the explant could also affect callus initiation of explants derived especially from a field grown plant (Dougall, 1977; Dixon, 1987; George and Sherrington, 1984).

Callus could be established from many explant type. Establishment of callus growth had been obtained from many *in vitro* plants species. According to Yeoman (1973), most viable plant cells could be induced to undergo mitosis from shoot tips or isolated meristems, which contained mitotically active cells for callus initiation and subsequent plantlet regeneration. The size and the shape of the initial explant was not critical, although proliferation might not occur with explants below a critical size. In general, fairly large pieces of tissues were

favoured because of the large numbers of cells present increased the chance of obtaining a viable culture. Therefore, a high surface area/volume ratio was desirable for a maximum growth (Yeoman, 1973).

According to Allan (1991), different plants required different nutrients, and callus could be derived from different plant parts and they required different nutritional constituents. A culture medium generally made up of inorganic and organic elements together with a carbon substrate and appropriate plant growth regulators. Plant growth regulators are important and the balance between auxin and cytokinin concentration is crucial in establishing callus cultures and maintaining them. However, some callus becomes habituated and they no longer require the addition of a particular plant growth regulator for their maintenance and growth. Mostly callus formed from the same explant can normally be grown on the same medium. A suitable medium for initiation and maintenance of callus can only be obtained by trial and error. The maintenance of cultures can determine whether a culture retains its organogenic potential (Allan, 1991). The most important factor in maintaining morphogenic potential is the tolerance of chromosome stability. The variation of chromosome number of plants in a long-term cell suspension culture has been well documented. Polyploidy and aneuploidy are a major source of somaclonal variation (Yeoman, 1973; Knorr & Dörnenburg, 1995).

2.2.3 Cell Suspension Culture

Plant cell cultures are initiated by transferring friable soft callus to liquid nutrient medium of the same composition as used for callus culture. Cell culture has to be agitated on an orbital shaker of between 90 and 150 rpm serves both

to aerate the culture and to disperse the cells. Kirsi and Wolfgang (2002) has stated that cell culture technique is an important system for genetic and breeding studies. Suspension cell cultures can also be used as the material of choice for biochemical and molecular investigation of plant secondary metabolite (Dicosmo and Misawa, 1995). The production of useful compounds by plant cell cultures has become increasingly significant especially the production of pharmaceutically important plant metabolites for the last few decades. Several compounds e.g., shikonin, berberine, and ginseng saponins have commercially produced from *in vitro* cell cultures. Scaling up from flask to bioreactor for the production of phytochemicals is always performed using suspension cultures (Eibl and Eibl, 2002). There are no specific conditions or procedure for cell suspension culture and the physical or biological conditions are determined by trial and error (King and Street, 1973).

There are several factors affecting secondary metabolite production using plant cell cultures such as plant growth regulators, medium nutrients, physical factors and biological factors. Extensive optimization studies in cultured plant cells are therefore necessary to increase cell biomass and production of bioactive compounds and this will indirectly reduce the production costs for future commercial production. For example, only 10% of *Catharanthus roseus* cells in culture actively accumulated anthocyanin, hence optimization to increase production of the anthocyanin become important (Yeoman, 1986).

Auxin and cytokinin affecting the production of secondary metabolite in plant cell cultures have been extensively investigated. It is well known that auxin

is essential and cytokinin is preferable to induce cell differentiation and to maintain cell proliferation *in vitro* (George and Sherrington, 1984). Gibberelin is usually not added to culture medium, and only a few reports describe its effect on natural product biosynthesis. Production of berberine in *Coptis japonica* cell cultures was increased by gibberelin (Yamada and Sato, 1981).

Cell density, which is mostly determined by cell inoculum size, is also an important factor affecting product yield. Optimum cell growth can be established with suitable inoculum size. It was reported that high density cell cultures were needed for ginseng saponin production from *Panax notoginseng* cell cultures (Zhang and Zhong, 1997) and anthocyanin production in *Perilla frutescens* cells (Zhong and Yoshida, 1995).

Sucrose concentration is one of the important factors in a plant cell culture. It is utilized as a carbon source. It was found that an increase in the sucrose concentration in a culture medium could result in an increase of secondary metabolite production (Dicosmo and Misawa, 1995). The enhancing effect of sucrose was most impressively shown in the case of rosmarinic acid formation in *Coleus blumei* cell suspension cultures, where the rosmarinic acid content increased six fold in medium containing 5 % sucrose compared with that in the control medium (2 % sucrose) (Petersen *et al.*, 1992).

Optimization of medium nutrients is also important to increase the productivity of particular secondary metabolites. There were a number of reports describing the effects of medium nutrients on secondary metabolites production

in plant cell cultures. One of the investigations was the manipulation of cell growth inhibition medium resulted an increase in the production of secondary metabolites, and the establishment of two stage culture system for production of phytochemicals. In this system, the cells were first cultured in a medium appropriate for maximum cell biomass production and then transferred to the growth limiting medium for maximum production of secondary metabolites. The production of anthocyanin from strawberry cell culture was established using the two stage cell culture system (Mayasuki *et al.*, 1998). Different concentrations of carbon (sucrose/glucose) and nitrogen (NO_3/NH_4 + ratio) were studied in *Azadirachta indica* (A. Juss) suspension culture and glucose was found to be a better carbon source over sucrose for yielding high cell biomass (6.32 g/L) and azadirachtin (11.12 mg/L) content. Nitrate alone as nitrogen source was favorable for both cell biomass and azadirachtin accumulation. (Prakash and Srivastava, 2005).

Physical factors such as light, temperature, medium pH, aeration rate, can also affect secondary metabolite synthesis in a cultured plant cells. The effect of light on secondary metabolite biosynthesis was found to be quite varied. Light illumination usually induced chloroplast differentiation which sometimes resulted in elevation of secondary metabolism production. Bioreactor cultured *Perilla frutescens* cells were able to accumulate significant amount of anthocyanin (about 10 % dry weight) without light irradiation when aerated at 0.2 vvm (Zhong *et al.*, 1993). Another biological factor that could influence secondary metabolite production was cell variation. Although cellular variation at molecular level affecting secondary metabolism had not yet been clarified, it had

been well recognized that selected cell aggregates with higher secondary metabolite content would eventually result in higher production of a particular secondary metabolite. Another biological factor was the stability of the biosynthetic capability of cultured plant cells. Alkaloid-producing cell lines of *Catharanthus roseus* that were established by repeated selection lost their biosynthetic ability during subcultures (Parr, 1988; Verpoorte and Memelink, 2002).

Elicitor was found to be effective in increasing the production of secondary metabolites. Cell culture of several plant species had been established for the production of secondary metabolites such as podophyllotoxin and its derivatives, glucoside, 5-methoxy-podophyllotoxin. Production cell culture of *Podophyllum hexandrum* was increased by a factor of 6- to 30-fold after the addition of the precursor Coniferyl alcohol, solubilized as a β -cyclodextrin complex or a glucoside from coniferin (Woerdenbag *et al.*, 1990). High producibility (0.2 % dry weight) of camptothecin was reported for *Catharanthus acuminata* with the addition of yeast extracts and jasmonates in the cell culture (Song and Byun, 1998). Putrescine treatment (0.1 mmol/L) influenced enhancement of growth and capsaicin production in the cell suspension cultures of *Capsicum frutescens*. On the other hand, the administration of polyamine inhibitor DFMA (α -DL-difluoromethylarginine) resulted in a reduction of cell growth, capsaicin content and the endogenous titres of polyamines (PAs). The capsaicin synthase activity was also higher in the putrescine (Put) treated cultures. Ethylene levels were lower in the cultures treated with putrescine. This study suggested that Put facilitates growth and

capsaicin production (Sudha and Ravishankar, 2003). Cell suspension cultures of *Cistanche deserticola* were established on Murashige and Skoog medium supplemented with 1.0 mg/L NAA, 2.0 mg/L BA, 0.25 mg/L 2,4-D and 30 g/L sucrose to produce phenylethanoid glycosides (PeGs) which had proven to possess free radicals scavenging activities, enhancing immune system, improving sexual function and sedative effect. PeGs production in *C. deserticola* cell cultures was improved by adding organic compounds and increased initial phosphate concentration in the culture medium (Cheng *et al.*, 2005). *Hypericum perforatum* L. (St. John's wort) is an herbal remedy widely used in the treatment of mild to moderate depression. Hypericin, a photosensitive naphodianthrone, is believed to be the compound responsible for reversing the depression symptoms. A dramatic increase in cell growth and hypericin production in *H. perforatum* cell cultures were observed after exposure to jasmonic acid (JA). However, other elicitors such as salicylic acid (SA) and fungal cell wall elicitors failed to show any stimulatory effect on either the cell growth or hypericin production of *H. perforatum* cell cultures (Walker *et al.*, 2002). Elicitors and precursors were optimized for paclitaxel production in cell cultures of *Taxus chinensis* in the presence of nutrient feeding. Paclitaxel production reached 54 mg/L (twice the amount without optimization) when 10 mg/L silver nitrate, 6mg/L abscisic acid, 23 mg/L chitosan, 15 mg/L phenylalanine, 31 mg/L methyl jasmonate, 30 mg/L sodium benzoate and 30 mg/L glycine were added on day 12 together with feeding solution containing 20 g/L sucrose added on day 16, which was twice the amount of paclitaxel produced without optimization (Luo and He, 2004). Chong *et al.* (2005) reported different elicitors and different day of treatment exerted different effects on cell growth and anthraquinone (AQ)