PRODUCTION OF JUVENILE HORMONE III (JH III) FROM *Cyperus aromaticus* VIA CELL SUSPENSION CULTURE TECHNIQUE AND ITS EFFECT ON MOSQUITO LARVA, *Aedes aegypti*

by

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LIST OF ABBREVIATION

MS	Murashige-Skoog
B5	Gamborg B5
BA	6-benzylaminopurine
NAA	1-napthaleneacetic Acid
IBA	3-indole butyric Acid
IAA	3-indole acetic Acid
ABA	Abscisic acid
2,4-D	2,4-dichlorophenoxyacetic acid
Picloram	4,amino-3,5,6-trichloropicolinic Acid
TDZ	Thidiazuron
2-iP	2-isopentenyladenine
PGR	Plant Growth Regulator
SE	Somatic embryo
NaOCl	Sodium Hypochloride
$HgCl_2$	Mercury Chloride
v/v	Volume per Volume
w/v	Weight per Volume
NaOH	Natrium hydroxide
HCl	Hydrochloric acid
ANOVA	Analysis of Varians
EC	Effective Concentration
s.d.	Standard division
s.e.	Standard error

ATCC	The American Type Cell Collection
NCI-H23	Lung cancer cell line
Caov-3	Ovarian cancer cell line
T-47D	Breast cancer cell line
$HgCl_2$	Mercuric chloride
NaDCC	Sodium dichloroisocyanurate
MBA	Methylene Blue Assays
MSB	MS supplemented with 271.1 mM NH_4NO_3 + 15.4 mM KNO_3 + 4.3 mM $CaCl_2.2H_2O$ + 1.49 mM $MgSO_4.4H_2O$ + 1.35 mM KH_2PO_4 + 3% sucrose + 5.5 mg/L NAA + 4.5 mg/L 2,4-D

PENGHASILAN HORMON JUVENIL III (JH III) DARIPADA *Cyperus aromaticus* DENGAN KAEDAH KULTUR AMPAINAN SEL DAN KESANNYA TERHADAP LARVA NYAMUK, *Aedes aegypti*

ABSTRAK

Kalus *Cyperus aromaticus* diaruh daripada eksplan akar menggunakan medium Murashige dan Skoog (1962) yang diubah suai dan ditambah dengan 5.5 mg/L NAA + 4.5 mg/L 2,4-D. Daripada kalus yang berasal daripada tisu akar, tujuh sel turunan yang stabil dipilih. Biojisim sel yang optimum dengan kandungan Hormon Juvenil III (JH III) yang tinggi dapat dihasilkan melalui sistem kultur satu peringkat mengunakan medium kultur yang diubah suai. Tempoh kultur optimum untuk penghasilan biojisim sel dan kandungan JH III yang tinggi ialah 12 hari. Medium kultur yang dioptimumkan telah diformulasi melalui pengubahsuaian makronutrien yang terkandung dalam medium MS asas dan pengawalatur pertumbuhan tumbuhan secara peringkat demi peringkat. Kesemua sel turunan terpilih tumbuh dengan baik di dalam medium optimum ini yang merupakan medium MS ditambah dengan 271.1 mM NH_4NO_3 + 15.4 mM KNO_3 + 4.3 mM $CaCl_2.2H_2O$ + 1.49 mM $MgSO_4.4H_2O$ + 1.35 mM KH_2PO_4 + 3% sucrose + 5.5 mg/L NAA + 4.5 mg/L 2,4-D medium (MSB). Penambahan 5.0 mg/L Chitosan® ke dalam medium MSB dapat menghasilkan JH III sebanyak 0.40 mg/g dalam 12 hari. Manakala, penambahan 1.0 g/L ekstrak yis ke dalam medium MSB dapat merangsangkan sel *C. aromaticus* menghasilkan dua kali ganda kandungan JH III berbanding dengan sel yang dikultur di dalam medium MSB tanpa ekstrak yis. Penambahan 0.3 g/L kasein hidrolisat ke dalam medium MSB menghasilkan kandungan JH III yang lebih tinggi, iaitu 1.50 mg/g dalam 12 hari. Ekstrak mentah sel *C. aromaticus* yang mengandungi JH III didapati memanjangkan kitaran hidup larva dan nyamuk dewasa *Aedes aegypti*.

Larva dan pupa menjadi abnormal selepas terdedah kepada ekstrak mentah *C. aromaticus*. Kesan residual dapat dilihat dalam generasi seterusnya. Terdapat penurunan bilangan telur yang dihasilkan daripada larva yang telah dirawat dengan ekstrak mentah *C. aromaticus*.

PRODUCTION OF JUVENILE HORMONE III (JH III) FROM *Cyperus aromaticus* VIA CELL SUSPENSION CULTURE TECHNIQUE AND ITS EFFECT ON MOSQUITO LARVA, *Aedes aegeptyi*

ABSTRACT

Callus of *Cyperus aromaticus* was induced from the root explants using modified Murashige and Skoog (1962) medium supplemented with 5.5 mg/L NAA and 4.5 mg/L 2,4-D. Seven stable cell lines were selected from these root-derived calluses. Cell suspension cultures were then established from these selected cell lines. Optimum cell biomass and high Juvenile Hormone III (JH III) content could be produced via single stage cell culture system using a modified culture medium. JH III of the tested samples was detected with high performance liquid chromatography (HPLC). The optimum culture duration for the production of high cell biomass and JH III content was 12 days. The optimized culture medium was formulated via step-wise modification of macronutrients portion of the basic MS medium and plant growth regulators. All the selected cell lines grew well in this optimum medium which consisted of MS supplemented with 271.1 mM NH_4NO_3 + 15.4 mM KNO_3 + 4.3 mM $CaCl_2.2H_2O$ + 1.49 mM $MgSO_4.4H_2O$ + 1.35 mM KH_2PO_4 + 3% sucrose + 5.5 mg/L NAA + 4.5 mg/L 2,4-D (MSB) medium. The addition of as low as 5.0 mg/L Chitosan® into the MSB medium could induce the production of JH III (0.40 mg/g) within 12 days. While the addition of 1.0 g/L yeast extract into MSB medium stimulated the *C. aromaticus* cells to produce twice the amount of JH III as compared to cells cultured in MSB without yeast extract. Addition of 0.3 g/L Casein hydrolysate into MSB medium was also able to induce higher production of JH III with 1.50 mg/g was produced within 12 days. Crude extracts of *C. aromaticus* cells

that contained JH III was found to extend the life cycle of *Aedes aegypti* larvae and mosquitoes. The larvae and pupae became abnormal after exposure to the crude extract of *C. aromaticus.* The residual effect also could be seen in the next generation. There was a reduction in the number of eggs produced by the mosquitoes developed from the larvae treated with crude extract of *C. aromaticus.*

CHAPTER 1

INTRODUCTION

1.1 Introduction

Cyperus aromaticus (Ridley) Mattf. and Kukenth, a perennial weed species, belongs to Cyperaceae family and grows naturally in tropical and temperate countries. The common name of *C. aromaticus* in English is 'Greater Kyllinga' (Henderson, 1954). It is also known as Navua sedge and 'rumput ganda' in Malaysia. It was originated from East Africa and brought to Asia from there (Hsuan *et al*., 1998). It can be found by the roadside and damp wasteland. It propagates very fast by rapid production of its rhizomes and tubers (Land protection, 2006). Most of the plants from the Cyperaceae dominate a new wasteland via vegetative propagation (Mansor, 1994). Other species in the Cyperaceae family that propagate very fast are *C. iria, C. asculatus, C. rotundus. C. digitus, C. elatus, Fimbristylis acuminate,* and *Lipocarpha argentea* (Commom Malaysian Weed and Control, 1972). *C. aromaticus* can grow to a height of 10 to 50 cm when mature. Its stem is solid triangular form and they are closely tufted together with purplish or reddish sheath at the base (Metcalfe, 1971).

Toong *et al*. (1988) successfully isolated the insect juvenile hormone lll (JH lll) from *C. iria* and *C. aromaticus*. The JH lll present in these plants were found to have the same 10 R configurations as that previously determined for JH lll secreted from the insect tissues. The Juvenile Hormone (JH) plays an important role in maturation, moulting and physiology processes in many insects (Bede *et al*., 1998). Insect juvenile hormones are critical developmental hormones that have direct effects on both larval development and adult reproductive competence. Most insect orders appear to synthesize a single JH homolog, the methyl (2E, 6E) 10,11-epoxy-

3, 7,11-trimethyl-2, 6-dodecadienoate (JH III) (Keiser *et al.,* 2002). Juvenile Hormones are a family of the closely related sesquiterpenoid group. The existence of Juvenile hormones in plants as secondary metabolites is for self-defence, and protect them from insect attack. The discovery of Juvenile hormones has resulted in much interest of using JHs as an insect control agent. JHs affect the development of internal organs, including the central nervous system, and promote the synthesis and release of sex attractant pheromones. They coordinate the regulation of the hemolymph ecdysteroid titer during pupal stage in *Manduca sexta* (Insect Biochem, 1987). It was found that the JHs and JH III blocked the growth of parasite, *Trypanosoma cruzi,* without killing the cells (Stoka, 1996).

Several insecticides have been used to control the mosquitoes and insects population. Long-term use of chemical insecticide created a lot of problems and also harmful to all living organisms and the environment. Dichlorodifenilrtrichloroetena (DDT) has been used as an insecticide and it is able to solve most of the problems caused by insects but it is toxin and resulted an unbalanced insect population. The active ingredient in DDT could remain active for a long period in the soil and this would affect the food chain (Speight *et al*., 1999). It kills not only the mosquitoes but also affects the population of other insects, fish and other organisms. According to Edwards (1994), the usage of synthetic insecticide and pesticide for a long period would be harmful to human, ecosystem and the environment.

The Insect growth regulator (IGR) has been suggested to be used as a bio-control for the mosquito population. JH III was found to control the development of insect from the nymph stage to adult. With high JH III, the mosquito larvae will not immerge to adult and it will remain at the larvae stage. It will also kill the larvae at

high concentration. Hence, JH III has a good potential to be used as a bio-insecticide and less harmful to environment and living organisms.

JH III can be extracted directly from *C. aromaticus* plants that are grown as weed in the field but this is not an efficient way of mass-producing the active compound as the environment factors can affect the consistent production of JH III in plant extract. *In vitro* culture techniques could hence be used as the alternative means for mass production of JH III via callus culture or the cell suspension culture techniques.

Using *in vitro* culture technique, the entire biotic and non-biotic factors such as weather, geographical, soil, humidity that can affect the secondary metabolite content in the plant can be controlled. This technique enables us to produce the plantlet and secondary metabolite in a clean environment that is free from microganisms such as bacteria and fungus. JH III was detected in the *in vitro* plantlet and callus culture of *C. aromaticus* by Chan *et al*. (2004).

Selection of elite cell line is important for the production of secondary metabolite and cell biomass. Luthfi *et al.* (2003) reported that different cell lines could produce secondary metabolite with increased cell biomass using a specific established cell culture system. Selected elite cell lines could produce high level of shikonin from cell suspension culture of *Lithospermum erythrorhizon* (Hara, 1996). Based on Oksman and Inze (2004), *in vitro* culture technique is one of the best alternatives for mass production of secondary metabolites from plant cells.

Dengue fever continues to be a major health threat in Malaysia since 1902 (Abubakar and Shafee, 2002). It is a mosquito transmitted disease and *Aedes aegypti* is the vector that transmits the viruses that cause the dengue fever and yellow fever. *Ae. aegypti* can be found at tropical and sub-tropical regions. Many researches have

been attempted to discover vaccines or drugs for treating dengue fever. However, until today there is no vaccine or drug that can control and treat the dengue fever. Fogging with chemical insecticide and clean up the entire compound are the only ways to prevent the wide spread of *Ae*. *aegypti* mosquitoes. Synthetic insecticides like organochlorin, organophosphate, carbonate and pyrethrod synthetic were used to control the insect (Yap *et al*., 2003). These chemical insecticides are harmful to the environment, flora and fauna including human. One of the alternatives to control the insect population and mosquito is to make use of the insect growth regulator (IGR) to regulate the development of insect during metamorphosis (Slama, 1971). The IGR can be used to control the insect population instead of using synthetic chemical to kill them. These IGR can be produced from the plants using cell suspension culture technique. Spilanthol, a secondary metabolite from *Spilanthes acmella*, was produced from the cell suspension cultures and was used as larvacide (Pitasawat *et al*., 1998).

1.2 Objectives

The objectives of this study are:

1. To establish the callus and cell suspension culture of *Cyperus aromaticus*
2. To select the elite cell line for the optimum production of cell biomass of *C. aromaticus*.
3. To optimize the production of JH III and determine the content of JH III from the cell suspension cultures of *C. aromaticus.*
4. To study the larvicidal activity of JH III towards *Aedes aegypti.*

CHAPTER 2

LITERATURE REVIEW

2.1 *Cyperus aromaticus* Mattf and Kukenth

2.1.1 Biological aspect

Cyperus aromaticus is also known as Nagua sedge or rumput ganda in Malaysia or Tuise Fiti in Samoan. The specific feature of *C. aromaticus* is that the plants have glums with tiny torns. It produces long stem in triangular section with the leaves arranged in three lines. The flower can be bisexual or unisexual.

Based on the International Code of Botanical Nomenclature (ICBN), *C. aromaticus* is placed in a taxonomic hierarchy as stated below:

Kingdom: Plantae

Division: Spermatophyta

Sub-division: Angiospermae

Class: Monocotyledoneae

Order: Glumiflorae

Family: Cyperaceae

Tribes: Cypereae

Genus: Cyperus

Species: aromaticus

C. aromaticus (Figure 2.1) is a monocotyledonous weed grows whole year round. It is rhizomatous sedge and assumes to be of no economy value. It belongs to Cyperaceae family. *C. aromaticus* has a 30 to 70 cm long straight and triangular shaped stem and sometime it can reach until two meter in length (Land Protection, 2006).

The inflorescences for all the *Cyperus* species are different. *C. aromaticus* has spikelet in spike shape, short and small. Glum is small at the base of the plant and is a bisexual plant. Each flower has three stamens and produces superior ovary. Whereas other Cyperus plants with big glum are unisexual with short filaments, three stamens and yellow coloured anthers (Norhayati, 1992). Ngu (2002) reported that the macro and microstructures of the vegetative and reproduction parts of *C. aromaticus* could be used to differentiate it from the other *Cyperus* species.

C. aromaticus does not have any economy value instead it affects the yield of other plant species. Some other plants are not able to grow well when this noxious weed is around because they are very competitive in term of nutrients, water, sunlight and carbon dioxide that is needed for photosynthesis.

2.1.2 Origin and distribution

Cyperus aromaticus is originated from tropical Africa and currently can be found in many countries such as Sri Lanka, Malaysia, Australia, Vanuantu, Solomonas and Fiji. It is normally found at wetlands of the tropical and temperate countries. They can grow not only at lowlands but also at high land (Gifford and Baker, 1995). There are about 80 genera and 4000 species of Cypereceae that can be found in wet and water log condition (Nather, 1990). According to Hatfield (1973), *C. articulatus* can be found in Jamaica and Turkey by the streamside. In Malaysia, *C. aromaticus* can be found along roadside, lowlands, highlands, wasteland and even at the drain side. There are 24 genera and 154 species of Cyperaceae found in Malaysia (Henderson, 1994). The common species of *Cyperus* are *C. rotundus, C. iria, C. papyrus, C.esculentu,* and *C. brevifolius*.

Figure 2.1: *Cyperus aromaticus* grow naturally as a weed at wasteland near the Universiti Sains Malaysia.

2.1.3 Beneficial uses

C. articulatus is considered as a medicinal plant. It was used to treat many illnesses such as headaches, migraine and epilepsy in some of the African countries (Ngo Bum *et al*., 2004; Schultes and Raffauf, 1990). It is also used to relief nausea, flatulence and vomiting during pregnancy. The decoction of rhizomes of *C. articulatus* was found to possess sedative properties in mice (Rakotonirina *et al*., 2001). The Egyptians used *C. papyrus* to make paper (Burkill, 1966). *C papyrus* was also used for decoration and for making containers. *C. rotundus,* a medicine plant, is used for the treatment of skin disease in China while the people in Cambodia use it for relieving high fever and treatment of malaria. It is used in India for deworming (Chopra *et al*., 1956). *C. aromaticus* is considered as a weedy plant without any economical value until the discovery of JH III from this plant. In Malaysia, *C. aromaticus* is used as animal feeds especially the goat (Noorhayati, 1992).

2.1.4 Potential Bio-insecticide

Since the discovery of JH III from plant, many researches have carried out studies on the possibilities of its uses as a bio-insecticide. Bede and Tobe (2000) investigated the potential allelopathic activity of this compound and the structurally related sesquiterpenoid farnesol. Juvenile hormones (JHs) are sesquiterpene derivatives that regulate both morphogenetic and reproduction development in insect (Bede *et al*., 2001; Osir and Riddiford, 1988). Schooley and Baker (1985) discovered that JHs were groups of five sesquiterpene similar to JH0, 4-MeJHI, (10R, 11S)-JHI, JHII and (10R)-JH III. JHs that can be found not only in insect, but also in Cyperus plants. JH III was extracted from *C. aromaticus* together with the

precursor, methyl (2E, 6E)-farnesoate (Figure 2.1) (Toong *et al*., 1988). JH III has a formula structure of methyl-10R, 11-epoxy3, 7,11-trimethyl 2E, 6E dodecadienoate.

Molting is a process that insects emerge from larvae to adult. JH III is needed during this process. There are two types of JHs, JH mimic and JH non-mimic. JH mimic is a synthetic hormone, which has a different molecular structure but the reaction mechanism is the same as the naturally occurring JH. The synthetic JH is suitable for controlling the mosquito population because they are stable even at sensitive environment but it is expensive compared to the conventional larvicide.

Ng (1999) reported that the JH III extracted from *C. aromaticus* and *C. iria* could be used as the commercial insecticide. The tested JH III was very stable but the JH III content in the plant was not consistent. Besides *C. aromaticus* and *C. iria,* JH III was also detected from other *Cyperus* species such as *C. pennatus* and *C. compressus* (Ng, 1987). Discrepancies in JH III levels in the plants were attributed to different species and environment conditions under which the plants were grown (Bede *et al*., 1999).

JH and JH analogs were found to affect the vitellogenin synthesis on the hemolymph and ovarian development in unfed adult female ticks in *Ornithodoros moubata* (Chinzei *et al*., 1991). Schwartz *et al*. (1998) reported the effect of feeding the *C. iria* leaves on the larvae of *Aedes aegypti* mosquito, and it was found that 90% of those fed exclusively on the *C. iria* leaves produced deformed wings and other morphological changes consistent with the development of abnormalities in the presence of excess JH. The adult female grasshoppers became infertile. However, Toong *et al.* (1988) found that the third stadium grasshopper nymphs, *Melanoplus sanguiipes,* fed on *C. iria* seedlings showed no different in growth.

Lengyel *et al*. (2007) presented the first data stating that recognition cues of a eusocial Hymenopterans were influenced by JH III and could thus play a major role in the regulation of the dynamic nature of social insect colonies of the African ant *Myrmicaria eumenoides*. The germination of lettuce seed treated with JH III was found to be delayed. It also inhibited the shoot growth of rice. JH III tested on several fungi was found to be not affecting the growth of the fungi (Bede and Tobe, 2000).

The plant extract of *Pyrethrum cinerariaefolium* has also been used as insecticide. The extract was found to be only harmful to the insect but safe for the environment and human (Wigglesworth, 1996). Plants from the Compositae family such as the *Spilanthes* spp. had been reported to contain insecticidal compounds (Broussalis *et al*., 1999). Arnason *et al*. (1989) reported that some plants contained chemical compounds that were toxic to insect pest or interfered with the life cycle of a particular insect pest. Based on studies carried out by Arnason *et al*. (1989) and Broussalis *et al*. (1999), the isolated compounds from these plants were biodegradable and did not leave any toxic residues in the environment. Plants that were reported to contain useful chemical compounds as potential bio-insecticides were *Azadirachta indica* (Meliaceae), *Chrysanthemum cinerariaefolium* (Compositae), *Spilanthes* spp. (Compositae), *A. excelsa* (Meliaceae), and *Cyperus* spp. (Cyperaceae) (Krishnaswamy *et al*., 1975; Borges-Del-Castillo, 1984; Staba and Zito, 1985; Jondiko, 1986; Toong *et al*., 1988; Arnason *et al.,* 1989; Jacobson, 1989; Khalid *et al.,* 1989; Wagner *et al*., 1999; Zakaria and Ali Mohd., 1994; Schwartz *et al*., 1998; Ng, 1999, Allan *et al*., 2002; Ang and Chan., 2003). These findings had encouraged many researchers to carry out research on botanical insecticides.

2.2 *In Vitro* Culture Techniques

2.2.1 Micropropagation techniques

Plant tissue culture technique is viewed as a potential means of producing useful plantlet that are difficult and slow to propagate by conventional methods. All the tissue culture techniques started with a small section or pieces of tissues called explants. The explants that are used to initiate a culture can be leaf, root, stem, shoot tip, sucker, meristem tissue, flower, seed and other parts of the plant. Plant tissue culture is also referring to the propagation of any part of the plant in an aseptic condition in an artificial medium that provides the full nutrient that is needed by a plant (Chawla, 2000). Plant tissue culture technique has been applied in commercial floriculture and horticulture industries for the production of disease free plantlets (Thorpe, 1990). For sustainable and mass production of good quality plantlets micropropagation technique is an invaluable tool. It can be employed for large-scale propagation of disease free clones and gene pool conservation (Rout *et al*., 2006).

Many plant species have been successfully propagated using the *in vitro* technique such as *Harpagophytum procumbens* and *H. zeyheri* (Levieille, 2002), *Eleusine indica* (Yemets *et al.,* 2003), *Platanus acerifolia (*Liu and Bao, 2003), *Eucalyptus globules* (Pinto *et al.*, 2002), *Rosa hybrida* (Singh and Syamal, 2001), *Lilium longiforum* (Nhut *et al*., 2001), *Spilanthes acmella* L. (Tan, 2004; Ang and Chan., 2003), *Pereskia granifolia* and *Pereskia Bleo* (Chuah, 2006), *Pandanus amaryllifolius* (Gaurab *et al*., 2004), *Limonium sinuatum (*Tomoko *et al.,* 2002), *Orthosiphon stamineus* (Lee and Chan., 2003), *Hyoscyamus niger* (Lai, 2003), *Cyperus aromaticus* (Chan, 2005; Zainah, 2005), Smooth Cayenne pineapple (Firoozabady *et al*., 2003), *Hylocereus undatus* Britton et Rose (Yasseen, 2002), *Lepidium sativum* Linn. (Pande *et al.,* 2002), Slash pine (*Pinus elliottii*) (Cai *et al.,*

2006), *Platanus acerrifolia* (Liu and Bao, 2003), *Ailanthus altissima* (Anderson *et al.,* 1983), *Adenophora triphylla* L. (Chen *et al.,* 2001), *Lilium specioum* (Chang *et al.,* 2000), *Tilia platyphyllos* (Chalupa, 2003), *Capsicum annuum* L. (Kim *et al*., 2008), *Ochreinauclea missionis* (Chandrika and Ravishankar**,** 2009), and *Typhonium flagelliforme* L. (Su *et al*., 2000).

2.2.2 Callus culture

Callus is an unorganized tissue formed by the division of plant cells that are induced in an explant cultured on culture medium with different level of plant growth regulators, cytokinin and auxin (Endress, 1994). There are different types of plant growth regulators commonly used in *in vitro* plant culture research. They are the auxins, cytokinins, gibberellins and abscisic acid. They play important role in many development processes in plants. Auxins such as 1-napthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D) and 4,amino-3,5,6-trichloropicolinic acid (picloram) and other naturally occur auxins like indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA) are widely used for induction of callus from plant tissues. Other than auxin, cytokininn is also used for callus formation and the most commonly used are N^6-Benzyl Adenine (BA), thidiazuron (TDZ) and N^6-2-isopentyl-adenine (2-iP). Cytokinins are phytohormones that influence many essential plant developmental processes including cell division and cell differentiation (Mok and Mok, 1977; Liu *et al.,* 1994; Haberer *et al*., 2002).

Huang and Chi (1988) and Hagen *et al*. (1990) indicated that picloram was a potent auxin for the initiation and maintenance of certain callus and suspension culture such as potato and banana cultures. Ketchum *et al.* (1995) reported that picloram was the best auxin for callus induction of *Taxus brevifolia*. Tisserat (1985)

stated that higher level of auxin was needed for callus induction compared to cytokinins. Fett-Neto *et al*. (1994) also found the amount of cytokinin used was lower than auxin in the callus culture of *Taxus cuspidate.*

Besides the growth regulator affect, types of explants and plant genotype could also affect callus formation. The induction of callus in woody plants is more difficult than the herbaceous plants. Monocotyledonous plants are more difficult to form callus than dicotyledonous plants (George and Sherrington, 1984). Many studies were carried out on the callus formation using the different plant parts. (Zheng *et al*., 1989) successfully induced callus formation from *Panax notoginseng* roots, tubers, stems, petioles, leaves, and flower buds. Leaf explants of *Echinacea purpurea* L. were used for callus induction using MS medium containing 2,4-D (Jones and Hang, 1977). While MS medium supplemented with combination of BA and IAA was found to be more suitable for the callus production from the leaf explants of *Phlox paniculata* (Jain *et al*., 2002), *Eurycoma longifolia* Jack (Luthfi *et al*., 2003), and *Orthosiphon stamineus* (Lee and Chan., 2003). Root explants were used for callus formation from *Cyperus aromaticus* (Chan, 2005), *Echinacea angustifolia* (Sandmith *et al*., 2002), *Hydrastic canadensis (*Hall and Camper, 2002) and *Podophyllum hexandrum (*Chattopadhyay *et al.*, 2002*).*

Callus can be grouped into friable, compact and pigmented type. Wu *et al*. (2003) reported that compact callus was induced from the stem of *Rhodiola sachalinensis* on MS medium supplemented with 0.5 mg/L NAA and 1.0 mg/L BAP whereas the fragile callus was induced on MS medium supplemented with 2 mg/L 2,4-D and 0.20 mg/L BAP. Chia *et al*. (1988) found that the calluses induced from *Hedyotis corymbosa* and *Hedyotis diffusa* leaf explants were green in colour with compact texture. Hence, different explant types and plant species cultured on media

supplemented with different plant growth regulator induced different texture and colour of callus.

2.2.3 Cell suspension culture

Cell suspension culture is established by inoculating the friable callus into a liquid culture medium and placed it on the shaker for continuous agitation to segregate the cells and increase the aeration of the cell culture. The cells usually proliferate faster and obtain more cell biomass than the callus culture due to the direct contact of the cells with the plant growth regulators and nutrients of the medium. Hence, many studies have been carried out to mass produce the plantlets and secondary metabolites using the plant cell culture technique.

Cultured plant cells are often believed to produce reduced quantities and different profiles of the secondary metabolite when compare with the intact mother plant. These could be due to the various biotic and abiotic factors that affect the production of the required and desired active compounds. However, Kamisako *et al.* (1993) and Cho and Kang (1997) reported that the cultured cells of cucurbitaceous plants, like *Cucumis melo* and *Luffa cylinfrica*, produced higher amount of triterpenoid identified as bryonolic acid than that produced from the roots of the mother plants. The culture medium nutrients such as the organic and inorganic salts were found to play an important role in maintaining a healthy cell suspension culture (Wetter and Constabel, 1982; Gamborg *et al.,* 1968). *Limonium sinuatum* Mill. cv. 'Early Rose', an important ornamental crop, was regenerated using the cell culture technique. High proliferation ability was obtained during two weeks subculture interval in half strength MS medium supplemented with 1.0 mg/L picloram (Tomoko *et al.,* 2002). Fki *et al.* (2003) established an improved protocol for high

proliferation in cell suspension culture of date palm (*Phoenix dactylifera* l., cv. Deglet Nour) with liquid medium supplemented with a lower concentration of 2,4-D and charcoal.

Cell proliferation with good cell biomass increases after subcultivation on a fresh culture medium and this is mainly due to the replenishes of the organic nitrates (Chungue *et al*., 1984). The amount of plant growth regulator such as auxin supplemented into the cell culture medium normally is lower than the solid medium for callus culture because the interaction between the cells and medium is higher in liquid medium. Higher concentration of auxin in the liquid culture medium could result in cell aggregation in the cell suspension culture (Dodds and Robert, 1995).

Cell culture technique is also frequently used in pharmaceutical fields to mass produce useful secondary metabolites like alkaloids, bryonolic acid, and terpeniod. Zenk and Wilhelm (1997) reported that the cell cultures of various *Berberis* species could produce alkaloid like isoquinolin, jatrorrhizin and berberin. The tropane alkaloids, atropine and scopolamine are widely used as anticholinergernic drug and they could be obtained from *Atropa belladonna* and *Hyoscyamus muticus* using cell culture (Collinge and Edward., 1985). Bisset (1982) reported that quinine was obtained from Cinchona bark using the cell suspension culture technique.

At perspective of pharmaceutical, Becerra-Arteaga and Shuler (2007) reported that glucosamine was produced after *in vitro* modification to yield a more human-like glycan and could serve as a route to produce therapeutic glycoproteins via plant cell culture. Lienard *et al*. (2007) showed that suspension-cultured BY-2 tobacco cells represented a low cost and environmentally safe expression system. This system could be applied to produce recombinant allergens from

Dermatophagoides pteronyssinus appropriate for diagnostic and therapeutic purposes. Using the plant cell suspension culture technique with the additional of methyl jasmonate, anthocyanins could be produced in the suspension cultures of *Vitis vinifera* (Qu *et al*., 2006). This indicated that cell suspension culture can be used not only to mass production of cell biomass but also secondary metabolites that can be used in pharmaceutical, diagnostic and therapeutic purposes.

2.3 Production of secondary metabolites

2.3.1 Selection of elite cell lines

In vitro plantlets, callus and cell cultures are usually used for selection of elite clones or lines. Elite plants were selected for sugar cane, paddy, tomato and banana based on high proliferation rate. Selection of elite lines usually is based on high proliferation biomass or high quality of the desired secondary metabolite (Ganapathi and Kargi, 1990). Singaram (2004) had selected elite clones of *Artemisia annua* for the production of artemisinin based on their growth rate. Luthfi *et al.* (2003) selected elite callus culture of *Eurycoma longifolia* based on its biomass. The selected elite callus cultures that produced high cell biomass were used as material source for the preparation of cell culture for the production of alkaloid 9-methoxycanthin-6-one and 9-hidydroxycanthin-6-on. Tabata (2004) obtained an elite cell line of *Taxus baccata* that could mass produce placitaxel and baccatin III in their optimized formulated medium.

Adam *et al*. (2003) reported that selection of a mutant cell line of *Nicotiana tabacum* could grow four times faster than the parental culture from which it was derived. This cell line had five-fold greater phosphomannose isomerase activity than the parental culture. Patnaik and Delbata (1997) reported that plantlets regenerated

successfully from the NaCl tolerant selected callus lines of *Cymbopogon martini* (Roxb.) Wat. had high percentage (70%) of survival after transferred to the green house.

Duration and frequency of subculture cycles could affect productivity of the selected cell lines. Zhao *et al*. (2001) reported that the texture of *Saussurea medusa* callus changed to yellowish white friable type of callus after 5-6 subculture cycles. Cultures that were kept for long duration without subculturing could affect the production of secondary metabolite due to accumulation of toxic waste from the cultures (Torres, 1989). Chen *et al.* (1995) reported that subculture cycles of seven and 10 days were suitable for the cell cultures of *Perilla frutescens* for active cell growth and production of a high level of anthocyanin respectively.

Cell lines selection not only used for the secondary metabolites production, it was also applied for the genomes and hybrid chromosome study. Koshinsky *et al*, (2000) reported that hybrid chromosome could be detected only in the selected cell line. The feasibility of site-specific recombination between genomes of different species offered new possibilities for engineering hybrid chromosomes that could be maintained in cell culture. Khosroushahi *et al*. (2006) developed a two-stage cell culture system for *Taxus baccata* L. using modified B5 medium in order to improve cell growth as well as productivity of secondary metabolites after callus induction and cell line selection for Taxol mass production.

2.3.2 Optimization for cell biomass and secondary metabolite production

2.3.2.1 Inoculums size

Inoculum size is one of the important elements in the establishment of plant cell suspension culture for the production of cell biomass and secondary metabolite.

A correct ratio of cell mass and medium volume must be used for optimum cell growth. Schwenk and Hildebrandt (1972) reported that the optimum plating density for cell culture was 1.5-5.0 x 105 cells per 60 mm x 15 mm plate. Inoculum size below or above this density, the cells would not grow properly.

The effects of inoculums size on polysaccharide synthesis in cell suspension culture of *Gardenia jasminoides* Eills were studied and an inoculum of 80g fresh cell in one liter medium was found to be the optimum size for cell growth and polysaccharide accumulation (Wang *et al.*, 2001). Zhong *et al*. (1995) reported that inoculum size did effect the kinetic of cell growth and secondary metabolites (anthocyanins) production of *Perilla frutescens* cell culture. The cell biomass and anthocyanin pigments produced were 3.3 and 24 times respectively using 15g/L fresh cell inoculum per liter medium compared to other inoculumn size. Lee and Shuler (2000) reported that high-inoculum-density (100 g FW/L) cultures produced higher ajmalicine concentrations compared to low inoculum density (50 g FW/L) but Catharanthine production was similar between the two different inoculum-density cultures. Chiou *et al*. (2001) found that optimal inoculum size and glucose concentration for polyunsaturated fatty acids (PUFA) production were 8-12% and 20-30 g/L respectively for the production of PUFA, arachidonic acid (ARA) and eicosapentaenoic acid (EPA) in *Marchantia polymorpha*.

2.3.2.2. Elicitor

Elicitation is the induction of secondary metabolite production by biotic and abiotic factors or treatment (Singh and Syamal, 2001). Some of the biotic treatments include fungal extract, enzymes, cell wall of microorganism (chitosan, gluycan) and bacteria (Darvill and Albersheim, 1984). While the abiotic elicitors commonly used

are heavy metal (Cd, Cu, Ag, Pb and etc), UV light and inorganic salts (Endress, 1994). The application of elicitors to plant cell and organ cultures is useful for enhancing the *in vitro* production of valuable secondary metabolites and cell biomass of some plant species such as *Gloriosa superba* (Buitelaar *et al.*, 1992; Ghosh *et al*., 2002), *Panax Ginseng* (Lu *et al*., 2009), *Salvia miltiorrhiza* (Chen *et al*., 2001), *Eurycoma longofolia* (Luthfi *et al.*, 2003), *Morinda elliptica* (Chong *et al.*, 2004), and *Decalepis hamiltonii* (Seemanti *et al*., 2006).

The concentration of the elicitor could affect the growth and the secondary metabolite production of the cells. High concentration of elicitors could cause cell mortality. The optimum concentration of elicitor that can be applied to any plant cell culture is depending on the plant species as different plant cells have different metabolism pathway, different response and different nutrient requirement. Tyler *et al.* (1988) had proven that high dosage of fungal homogenate (biotic elicitor) increased cell mortality of *Papaver somniferim* and the cells changed to dark colour. Lufthi *et al.* (2003) reported that even the day of applying the elicitor had affected the growth and alkaloid production from the *Eurycoma longifolia* cell culture. Chai *et al*. (2002) reported that low-energy ultrasound (US) could affect the plant defense responses and secondary metabolite production from the *Panax ginseng* cell suspension culture. It stimulated the synthesis of useful secondary metabolites, saponins, without causing any net loss of the ginseng cell biomass.

The trivalent ion lanthanum, a rare earth element, was tested for elicitor-like effects on taxol production in cell suspension cultures of *Taxus* spp. The maximum stimulation of taxol production was achieved with the addition of 5.8 μM La^{3+} to the culture during mid-log growth phase. The addition of La^{3+} has increased the taxol yield by nearly three-fold over a 28 days culture period. At higher concentrations,

the lanthanum ion caused cell growth inhibition (Wu *et al*., 2001). Chong *et al*. (2004) reported that *Morinda elliptica* cell cultures treated with jasmonic acid on 12 days old cells increased 2-fold production of anthraquinone.

2.3.2.3 pH of culture medium

In plant cell culture, pH is an important factor influencing the yield of cell biomass and secondary metabolite accumulation. The hydrogen ion concentration in the culture medium was due to the chemical nutrients such as nitrate and ammonium. The change in H^+ ions could affect the cell growth and secondary metabolite production in plant cells (Endress, 1994). The culture medium constituents such as the macronutrient, micronutrient, sucrose, and plant growth regulators could change the pH of the medium (Gunawan, 1988; Minocha, 1987). The pH of most of the plant cell culture medium was adjusted to between five to seven. The pH of the culture medium can affect the ion uptake and at the same time affect the existing ionic competitions between proton, cation, and anions that are important for the plant nutrient (Mengel *et al*., 1984; Marschner *et al.*, 1995). Suzuki (1995) found that secondary metabolites could be released from the cells with reducing pH of the medium. Martin and Rose (1977) reported that the pH of the cell suspension culture medium affected the cell biomass *Ipomoea* sp. The cell biomass was low at pH 4.8 and 7.2 compared to the intermediate pH levels in the medium.

Acidic medium generally could not be used for cell culture of all the plant species. It also affects other *in vitro* cultures beside cell cultures. Huang and Chou (2006) reported that the pH level affected the growth of hairy roots of *Stizolobium hassjoo* in B5 medium. When the pH level declined towards 4.9, the growth of hairy roots was very slow and it can be conceived as a preparatory period of cell division.

From their discovery, pH affected the uptake of NH_4^+, NO_3^- , and PO_4^- .These would affect the cell membrane and thus favored the subsequent root growth. However, Sandra *et al.* (2000) reported that a decrease in the culture medium pH did not affect the growth of the transformed roots of *Brugmansia candida* for the production of hyoscyamine and scopolamine.

Besides affecting the growth of plant cells, the pH of the culture medium can also affect the whole organ and organ cultures. Gabriella *et al.* (2002) reported the effect of medium pH on the ion uptake in *in vitro* vegetative organogenesis of tobacco (*Nicotiana tabacum* L. Cv. Samsun). The *in vitro* shoots were developed normally in medium with initial pH of 4.0 and 5.0 and the shoots grew into small clusters at pH 7.0 after 25 days of culture. This indicated that pH of the nutrient solution strongly influenced the ion uptake and vegetative organogenesis of Tobacco.

2.3.2.4 Carbohydrate content

Plant carbohydrate is one of the main sources for plant growth and secondary metabolite production. Carbohydrates in the medium are used for the development of cell vacuoles and thickening of cell wall for accumulation of the polysaccharides, enhance cell growth and increase the yield of secondary metabolite in the cell cultures (Endress, 1994). For example, high sucrose (45 g/L) was favourable for the production of anthocyanin in the cell culture of *Perilla frutescens* (Zhong *et al.,* 1995). Lufthi (2004) reported maximum cell growth with high cell biomass and high alkaloids production (9-hydroxycanthin-6-one and 9-methoxycanthin-6-one) with culture medium containing 4% sucrose. The amount of carbohydrate needed depending on the plant species. Zheng *et al* (1989) obtained the maximum cell

growth for *Panax ginseng* using medium containing 3% sucrose while mass production of saponin was achieved by using 6 to 8% sucrose supplemented into the culture medium. Swedlund and Locy (1993) reported that sucrose was a suitable and inexpensive carbohydrate source and could support an optimum cell growth. Other sugars such as glucose, fructose, glycerol and galactose have also been used as the carbon source for *in vitro* plant tissue and cell cultures of many plant species (Swedlund and Locy, 1993; Spiegel-Roy and Saad, 1997). The effects of medium and culture condition on polysaccharide synthesis in the suspension culture of Gardenia *jasminoides Eills* were studied by Wang *et al*. (2001). It was found that glucose was better than sucrose for cell growth, but due to the higher price of glucose, 45 g/L of combined sucrose with glucose (1:1) was used for optimum cell growth. However, high sucrose content could increase the cell biomass and secondary metabolite production but it also caused phenolic compounds accumulation in the apple shoot culture (Astrid *et al*., 2000), cherry culture (Treutter and Feult, 1998) and cell culture of strawberry (Mori and Sakurai, 1994). To enhance production of sakosaponin in the root culture of *Bupleurum falcatum* L., a two-step supplemented control of sugar was used with 1% sucrose at the beginning of the culture and addition of 6% sucrose on the 14th day of culture (Kusakari *et al*., 2000).

2.3.2.5 Macronutrient of medium

The aspects of other factors such as light, plant growth regulators, temperature, and medium content especially the macronutrient and micronutrient on the cell growth and secondary metabolite production from *in vitro* cells were studied for more than 30 years ago (Leifert *et al*., 1995). Macronutrient salts play an

important role in plant growth. Murashige and Skoog (1962) basic medium is commonly used in many plant tissue, callus and cell cultures until today such as *Panax ginseng* (Choi *et al.*, 2003), *Manihot esculenta* Crantz (Danso and Ford-Lloyd, 2004), *Hyoscyamus niger* (Lai, 2003), *Eurycoma longifolia* Jack (Luthfi *et al.*, 2003; Ang. 2007), *Cyperus aromaticus* (Chan *et al.*, 2004; Zainah, 2005; Choo, 2006; Che, 2002), *Azadirachta indica* (Kaveti, 2003), *Podophyllum hexandrum* (Saurabh *et al.*, 2002), *Rollinia mucosa* (Solange *et al.*, 2000), *Cucumis sativus* (Konstas *et al.*, 2003), and *Sphilanthes acmella* (Tan *et al.*, 2004; Ang and Chan, 2003). Different culture media have different level of macronutrient. Torres (1989) reported that some media have higher macronutrients content like Murashige and Skoog (MS), Schenk and Hildebrandt (SH) and Gamborg 5 (B5) if compared to other medium such as White medium.

The macronutrients supply magnesium, sulphate, sodium, potassium, and calcium elements and the nitrogen source. The optimum requirement of the macronutrient for each plant species is influenced by some other factors such as pH of the medium, carbohydrate content, temperature, physiological and biochemical status of the plant. The cell suspension culture of soybean (*Glycine max* L.) grew well in a defined medium with the nitrogen sources came from nitrate (25 mM) and ammonium (2 mM). The cells did not grow in medium containing only nitrate. The medium had to be supplemented with ammonium salts. Cell cultures of *Reseda luteoli* L., *Triticum monococcum* L., flax, *Linum usitatissimum* L., horseradish *(Amoracia lapathifolia* Gilib) and *Haplopappus gracilis* L. grew well on the B5 medium with nitrate (25 mM) as the sole nitrogen source. Higher cell yields were obtained when ammonium (2 mM) or glutamine were also supplemented into the cell cultures (Gamborg, 1970). The sapogenin steroid content increased significantly

in the *in vitro* cultures of *Agave amaniensis* with higher concentration of magnesium, copper and other ions in the culture medium (Andrijany *et al*., 1999). Weathers *et al.* (1994) found that the different level of ammonium and nitrate and the addition of GA3 in the B5 medium affected the artemisinin content of the *Artemisia annua* cells.

2.3.3 Extraction of secondary metabolites

Different solvents are needed to extract different secondary compounds. It depends on the types of compounds such as alkaloid, terpenoid, lipid and whether they are volatile or non-volatile compounds. To extract the alkaloids from *Eurycoma longifolia* cells, Luthfi (2004) used methanol to extract them at room temperature. Methane was also used to extract anthocyanin from *Daucus carota*, grape (Elizabeth *et al*., 2003), *Albizia gummifera, Pentas longiflora, Pittosporum lanatum,* (Wanyoike *et al.,* 2003), saikosaponins from *Bupleurum falcatum* (Kusakari *et al*., 2000), jaceosidin and hispidulin from *Saussurea medusa (*Zhao *et al*., 2004), isoflavonoid from *Lupinus albus* L. (Hubert *et al*., 1997), phenylethanoid glycosides from *Cistanche deserticola* (Cheng *et al*., 2005), sesquiterpene from *Hyoscyamus muticus* (Edgard and Wayne, 2002) and silymarin from *Silybum marianum* (Maria , 2005).

However, chloroform was used as solvent to extract the sesquiterpenoid and Juvenile hormone III from *Cyperus aromaticus* and *Cyperus iria* (Toong *et al.,* 1988; Schwartz *et al.,* 1998; Chan *et al.,* 2004; Zainah, 2005). The sesquiterpene, tessaric acid derived from *Tessaria absinthioides* (Kurina *et al*., 2000), shikonin from *Lithospermum erythrorhizon* (Touno *et al.,* 2000), Citronellal from *Cymbopogon nardus* L. (Chan *et al.,* 2005), were also extracted using Chloroform as