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SELECTION OF *Artemisia annua* L. CELL LINES FOR PRODUCTION OF CELL BIOMASS, ARTEMISININ AND DIHYDROARTEMISININ

by

CH'NG SONG JIN

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LIST OF ABBREVIATIONS

MS	Murashige and Skoog
BAP	6-Benzylaminopurine
NAA	1-napthalenenacetic acid
GI	Growth Index
HPLC	High performance liquid chromatography
UPLC	Ultra performance liquid chromatography
TLC	Thin layer chromatography
PDA	Photodiode array
rpm	Rotation per minute
ANSI	American National Standard Institute
AR	Analytical reagent
ANOVA	Analysis of variance
R^2	R-Squared
v/v	Volume per volume
w/v	Weight per volume

PEMILIHAN SEL TURUNAN *Artemisia annua* L. UNTUK PENGHASILAN BIOJISIM SEL, ARTEMISININ DAN DIHIDROARTEMISININ

ABSTRAK

Artemisia annua, tumbuhan herba aromatik, didapati menghasilkan artemisinin yang telah dibukti mempunyai sifat anti-malaria. Kultur kalus *A. annua* telah berjaya ditubuhkan dengan menggunakan eksplan daun daripada anak pokok *in vitro* melalui pengkulturan dalam medium asas MS yang ditambahkan dengan 0.5 mg/L BAP, 0.5 mg/L NAA dan 0.5 g/L kasein hidrolisat. Biomas kalus yang diinduksikan daripada klon dTC2 (0.968 ± 0.067 g) adalah lebih tinggi secara bererti daripada klon dTC1 (0.401 ± 0.098 g) dalam tempoh masa lima minggu. Kalus yang diinduksikan daripada klon dTC1 adalah putih, lembut dan melekit manakala kalus daripada klon dTC2 adalah hijau and rapuh. Proses pemilihan telah dijalankan dengan menggunakan kalus yang diperolehi daripada klon *A. annua* TC1, TC2, Highland, dTC1 dan dTC2 berasal dari Vietnam. Sejumlah 67 sel turunan telah dihasilkan daripada lima klon iaitu 15 daripada TC1, 10 daripada TC2, 20 daripada Highland, 12 daripada dTC1 and 10 daripada dTC2. Walau bagaimanapun, hanya sembilan sel turunan dipilih daripada TC1, enam daripada TC2, lapan daripada dTC1, tujuh daripada dTC2 dan empat daripada Highland berdasarkan kestabilan pertumbuhan sepanjang 13 kitar pengsubkulturan. Sel turunan yang dipilih dibahagikan kepada kategori pertumbuhan cepat dengan indeks pertumbuhan (GI) > 20, pertumbuhan sederhana (15≤ GI ≤20) dan pertumbuhan lambat (GI <15). Tiada sel turunan dengan pertumbuhan lambat dihasilkan daripada klon TC2 and dTC2 kerana kedua-dua klon ini adalah penghasilan biomas tinggi secara semula jadi. Ciri morfologi kalus

didapati mempengaruhi pertumbuhan sel turunan *A. annua*. Sel turunan dengan keamatan warna yang rendah menghasilkan lebih banyak sel biomas. Kebanyakan sel turunan rapuh termasuk dalam kategori pertumbuhan cepat. Pertumbuhan beberapa sel turunan (L2TC1, L9TC1, L1TC2, L4TC2 L1dTC1, L4TC1 dan L6dTC1) menjadi tidak stabil dalam tempoh pemanjangan kitar pengsubkulturan. Penghasilan artemisinin didapati tidak konsisten dalam kebanyakan sel turunan. Hanya dua sel turunan yang terpilih (L2TC2 dan L3dTC2) didapati menghasilkan artemisinin secara konsisten dengan kandungan lebih daripada 80 μg/g. Dihidroartemisinin, suatu terbitan daripada artemisinin, juga boleh dikesan dalam sel-sel turunan. Walau bagaimanpun, kandungannya adalah sangat rendah. Semua sel turunan mengekalkan keupayaan pertumbuhan dalam kultur ampaian sel kecuali sel turunan yang diterbitkan daripada klon Highland. Sel turunan rapuh didapati menghasilkan biomas tinggi dalam kultur ampaian sel. Walau bagaimanapun, sel turunan L8dTC1 yang padat juga didapati menghasilkan biomas yang tinggi tetapi ia tidak berkekalan dalam kultur ampaian sel. Sesetengah sel turunan (L13Hi, L12Hi, L1dTC1 dan L2dTC2) hilang keupayaan untuk biosintesis artemisinin dalam kultur ampaian sel. Kandungan artemisinin tinggi didapati dalam kultur ampaian sel L8dTC1, L3dTC1 dan L9dTC2 dengan kandungan artemisinin lebih daripada 200 μg/g. Kandungan dihidroartemisinin adalah rendah berbanding dengan kandunngan artemisinin dalam kultur ampaian sel kecuali sel turunan L8dTC1 yang menghasilkan dihidroartemisinin yang paling tinggi berbanding dengan sel turunan lain. Kesimpulannya, morfologi sel turunan boleh digunakan sebagai kriteria untuk pemilihan sel turunan berdasarkan biomas sel. Tetapi, ia tidak boleh dikaitkan dengan kandungan artemisinin dan dihidroartemisinin.

SELECTION OF *Artemisia annua* L. CELL LINES FOR PRODUCTION OF CELL BIOMASS, ARTEMISININ AND DIHYDROARTEMISININ

ABSTRACT

Artemisia annua, an aromatic herbaceous plant, was found to produce artemisinin that had proven to have antimalarial properties. Callus culture of *A. annua* was successfully established from the leaf explants of *in vitro* plantlets by culturing onto MS basal medium supplemented with 0.5 mg/L BAP, 0.5 mg/L NAA and 0.5 g/L casein hydrolysate. Callus biomass induced from dTC2 clone (0.968 ± 0.067 g) was significantly higher than dTC1 clone (0.401 ± 0.098 g) after five weeks of culture. The callus induced from dTC1 clone was white, soft and sticky while callus derived from dTC2 clone was green and friable. Selection process was carried out from the callus derived from TC1, TC2, Highland, dTC1 and dTC2 clones of *A. annua* of Vietnam origin. A total of 67 callus cell lines were produced from the five clones of which 15 lines were derived from TC1, 10 from TC2, 20 from Highland, 12 from dTC1 and 10 from dTC2. However, only nine lines were selected from TC1, six lines from TC2, eight lines from dTC1, seven lines from dTC2 and four lines from Highland based on the growth stability throughout 13 subculture cycles. The selected callus cell lines were categorised into fast growing with growth index (GI) > 20, intermediate (15≤ GI ≤20) and slow (GI < 15) growing group. There were no slow growing callus cell lines derived from TC2 and dTC2 clones as the two clones had high biomass productivity in nature. Morphological characteristic of the callus were found to influence the growth of the callus cell lines of *A. annua*. Callus cell lines with low colour intensity produced higher cell biomass. Most of the friable

callus cell lines were fallen into fast growing category while compact and spongy callus cell lines were fairly distributed into the three categories. The growth of several callus cell lines (L2TC1, L9TC1, L1TC2, L4TC2 L1dTC1, L4TC1 and L6dTC1) became unstable during prolong subculture cycles. Production of artemisinin was found to be inconsistent in most of the callus cell lines. Only two selected lines (L2TC2 and L3dTC2) were consistently produced more than 80 µg/g of artemisinin. Dihydroartemisinin, a derivative of artemisinin, was also able to be detected in the callus cell lines. However, the content was comparatively low. All the cell lines retained its growth ability in cell suspension culture except cell lines derived from Highland clone. Friable cell lines were found to produce higher biomass in cell suspension culture compared to non-friable lines. However, compact L8dTC1 line also found to produce high cell biomass but it was not sustainable in cell suspension culture. Some of the cell lines (L13Hi, L12Hi, L1dTC1 and L2dTC2) lost its ability to biosynthesis artemisinin in cell suspension culture. High artemisinin content was found in cell suspension of L8dTC1, L3dTC1 and L9dTC2 in which the content was more than 200 µg/g of artemisinin. Dihydroartemisinin content was low compared to artemisinin in the cell suspension culture except L8dTC1 line produced the highest dihydroartemisinin among the other cell lines. In conclusion, the morphology of the callus culture could be used as the criteria for selection of cell lines based on cell biomass. But, it did not correlate with the artemisinin and dihydroartemisinin content.

CHAPTER ONE

INTRODUCTION

Malaria, a serious global health problem, is caused by a parasite that is transmitted into human body through the bites of infected mosquitoes. According to statistic data, there are an estimated 655,000 deaths out of 216 million cases of malaria reported in 2010. Most death occur on children lived in Africa and an estimated 22 % of the childhood death is due to malaria. At present, approximately 3.3 billion people are at risk of malaria (WHO, 2011a). This disease is still endemic in more than ten countries in Western Pacific Region (WHO, 2011b) which related with poverty as this disease depressed the socio-economic development of the affected country (Gallup and Sachs, 2001).

The parasites that cause human malaria are mainly *Plasmodium vivax*, *P. falciparum*, *P. malariae* and *P. ovale*. They were transmitted to human body through the bites of mosquito vector, the *Anopheles* group (Lambert, 2003). *P. falciparum* and *P. vivax* were the most widespread parasites in malaria endemic country. The most deadly parasite was *P. falciparum* which caused most death cases across sub-Saharan Africa (Price *et al.*, 2007). Transmission of malaria parasite from Old World monkeys known as *P. knowlesi* was brought into attention by reported malaria cases in the Kapit Division of Sarawak state, Malaysia (Singh, 2004). This parasite was widely distributed in Malaysian Borneo and also Penisular Malaysia in which it was misidentified as *P. malariae* microscopically. With the used of nested-PCR assay, DNA of *P. knowlesi* was found on the blood sample of the four fatal cases in Malaysia (Cox-Singh *et al.*, 2008). This parasite was also found distributed in China, Indonesia, Philipines, Singapore, Thailand and Taiwan (Sabbatani *et al.*, 2009).

The malaria parasites became life threatening once it transmitted into human body. These parasites actively multiply in the liver and disrupt the blood supply to important organs by destroying the red blood cells (WHO, 2012a). Hence, antimalarial drugs were introduced especially to the endemic region of malaria to eliminate these parasites. However, only limited drugs can be used to treat malaria. The most commonly and widely used drugs were quinine and its derivatives (such as chloroquine). Besides these, other antimalarial drugs were antifolate combination drugs, antibiotic (such as tetracycline and clindamycin), and artemisinin compounds such as artesunate, artemether and arteether (Bloland, 2001). However, the effectiveness of the antimalarial drugs was affected by the evolution of the resistant strains of *Plasmodium* parasites. More than a decade ago, *P. falciparum* had become resistant to most of the current antimalarial drugs especially chloroquine. In some region, *P. vivax* also showed resistant to chloroquine (Murphy *et al.*, 1993).

The development of antimalarial resistant strains parasites was due to mono-drug treatment of malaria. Isolation of single malaria was found to be made up of heterogeneous populations of parasites in which their sensitivity towards antimalarial drugs were different. Prolong using of the mono-drug treatment resulted in increasing tolerance of certain populations of malaria parasites and development of resistant strains (Thaithong, 1983). Misdiagnosing and inaccurate drugs intake was also one of the factors that caused development of drug resistant strains of parasites (Wernsdorfer, 1994). Some strains of parasites especially *P. falciparum* were able to adapt rapidly when new drugs were applied even the drugs were not chemically similar (Rathod *et al.*, 1997).

The antiplasmodial therapy especially quinine-based therapies became ineffective for the malaria treatment due to the wide spread of multidrug resistant

Plasmodium parasites (White, 2004). Artemisinin-based combination therapies (ACTs) were the most effective antimalarial drugs currently available. ACTs were used for the treatment of severe malaria because of its rapid parasite clearance and ability to lower the rate of resistance emerges and spreads of malaria parasites (White, 1999; Mutabingwa, 2005). ACTs was designed with the combination of two effective drugs that had different clearance activity to enhance the effectiveness of the treatment of chloroquine resistant parasite strains. There was rising concern with the emergence of artemisinin resistant parasites detected along the borders between Thailand-Cambodia and Thailand-Myanmar (Noedl, 2008; Yeung *et al.*, 2009; WHO, 2012b). However, the efficacy of ACTs along the borders of Thailand remained high (Vijaykadga *et al.*, 2006). In spite of these, artemisinin and its derivatives are still the most effective drugs for treatment of malaria.

Although ACTs were efficient in treatment against most of the malaria parasites, the price was generally more expensive as compared to chloroquine. This is because the production process of artemisinin is complicated due to its chemical structure (endoperoxide sesquiterpene lactone) and not feasible for large scale production via chemical synthesis (Abdin *et al.*, 2003). Since the chemical synthesis is not suitable for large scale production, artemisinin is obtained mainly from the natural source of *Artemisia annua* plants.

A. annua or "qinghao" was traditionally used to treat intermittent fever. It was found to produce artemisinin (Klayman, 1985). However, the production of artemisinin from field grown *A. annua* plant was affected by environmental condition, geographical location and time of harvesting (Woerdenbag *et al.*, 1994; Gupta *et al.*, 2002; Thu *et al.*, 2011). The artemisinin production from the plant was also found to be affected by the genetic varieties of the plant. Different varieties or

clones produce varying amount of artemisinin. Therefore, it is important to select the high yielding varieties for production of artemisinin. One of the selected *Artemisia* hybrids (*Artemis*) was reported to produce up to 1.4 % artemisinin (Fereirra *et al.*, 2005). An alternative way to obtain this bioactive compound is via *in vitro* culture methods which are independent of environmental condition and geographical locations. Many researchers have reported the potential of producing artemisinin via *in vitro* culture techniques using shoot culture (Liu *et al.*, 2003; Lualon *et al.*, 2008), hairy root culture (Wang and Tan, 2002), and cell suspension culture (Tawfiq *et al.*, 1989; Liu *et al.*, 1992; Chan *et al.*, 2010). It was reported that feeding the *A. annua* cell suspension cultures with precursors and elicitors could enhance the production of artemisinin (Baldi and Dixit, 2008; Durante *et al.*, 2011).

Although *in vitro* culture methods are independent of environmental, ecological and climatic conditions for the production of artemisinin, but the inconsistent growth of the *in vitro* cultures and productivity is one of the major factors for concern (Kim *et al.*, 2004 and Qu *et al.*, 2006). Plant cell suspension cultures often cause genetically instability in term of growth and production of bioactive compounds due to somaclonal variation (Chattopadhyay *et al.*, 2002). Therefore, selection of high productive lines for initiation of suspension culture is important.

1.1 Objectives

To ensure sustainable and consistent production of artemisinin from the *in vitro* culture of *A. annua*, this study was carried out with the following objectives:

1. To obtain stable callus cell lines and to select elite lines via frequent subculturing of callus culture.

2. To correlate the cell biomass with the morphology of the *A. annua* callus.
3. To correlate the plant cell growth with the production of artemisinin.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Artemisia annua*

2.1.1 Backgrounds

Artemisia annua, an aromatic herbaceous plant, has been used as traditional Chinese medicine to treat diseases associated with fever and malaria for over 20 centuries (Ferreira *et al.*, 1997). This plant is known with various names in different regions such as sweet wormwood or annual wormwood in English, Qinghao in Chinese, Thanh cao hoa vàng in Vietnamese, Gae-tong-sook in Korean and Kusoninjin in Japanese (WHO, 2006). *A. annua* is categorized into Asteraceae family as shown in the taxonomy table below based on PLANTS database (USDA-NRCS, 2012).

Kingdom:	Plantae
Subkingdom:	Tracheobionta (Vascular plants)
Superdivision:	Spermatophyta (Seed plants)
Division:	Magnoliophyta (Flowering plants)
Class:	Magnoliopsida (Dicotyledons)
Subclass:	Asteridae
Order:	Asterales
Family:	Asteraceae (Aster family)
Genus:	*Artemisia* (sagebrush)
Species:	*Artemisia annua* L.

The genus name of *Artemisia* is named after the Greek goddess Artemis, daughter of Zeus. The derived name means the ability to heal and cure diseases (Guirand, 1959). The plants from the genus *Artemisia* had been used to relieve pain during childbirth, to induce abortion and to regulate menstrual disorder in ancient time (Riddle and Estes, 1992). *A. annua* is bitter in taste but cool in nature, hence it is usually used to treat fever related diseases such as jaundice, tuberculosis, and fever caused by summer heat or deficiency of "yin". It is also used to relieve headache, dizziness and nosebleeds (The Republic of China, 1985). Apart from treating diseases, it was used as fumigant insecticide to kill mosquitoes by burning the leaves of this herb (Foster and Chongxi, 1992).

During 1950s, due to the emergence of resistant strains of malaria parasites (*Plasmodium falciparum*) against chloroquine (the only existing antimalarial at that time), a group of researchers embarked on the finding of Chinese herbs that could used as new remedy for the treatment of the resistant strains of *P. falciparum*. They had found the extract of *A. annua* showed excellent results against the growth of the parasite. This herb became valuable when the active ingredient, artemisinin, was successfully identified in 1972 as reviewed by Tu (2011).

A. annua was originated from China particularly at the northern part of Chahar and Suiyuan province where the plant grows naturally as steppe vegetation (Wang, 2011). It is also widely distributed in Asia especially in temperate, cool temperate and subtropical zones (Ferreira *et al.*, 2005). This plant is now grown wild in many parts of the world such as middle, eastern and southern part of Europe (mainly in Bulgaria, France, Hungary, Romania, Italy, Spain and former Yugoslavia), United State of America, South America such as Argentina and country in North Africa (Klayman, 1989). Although the geographical range or relatively temperate latitude is

the main criteria in determination of the cultivation area for *A. annua*, it is successfully introduced in several regions at the lower tropical latitudes (Woerdenbag *et al.*, 1994). The plants were propagated and adapted well in these cultivation regions (Magalhaes and Delabays, 1996). However, China and Vietnam were the largest cultivation of *A. annua* for the production of artemisinin (Ferreira *et al.*, 2005).

2.1.2 Plant morphology

A. annua is an annual herbaceous plant which can grow up to 100 cm in height in the wild. Under proper cultivation, this plant can grow up to two metre high. It is usually single-stemmed with many alternate branching (Figure 2.1a). The stem is erected and ribbed (Figure 2.1b). It is green in colour at early growing stage and slowly changing to deep reddish at later development stage when starts branching (Polyakov, 1995). The number of the branching could reach up to 70 branches and the length of the branch may reach 200 cm (Thu *et al.*, 2011).

A. annua has aromatic, green and fernlike leaves. The leaves are fine pinnately dissected into very narrow lobes. These lobes are further dissected into fine dentate shape (Figure 2.1c). The leaf is alternately placed to each other and can reach 2.5 to 5 cm in length. The central vein of the leaf can be clearly seen and both side of the vein have deep and narrow pinna (Griffee and Diemer, 2005).

A. annua is a dicotyledonous plant with tap root system. The tap root is short but dense with aggressive lateral roots. This is because *A. annua* needs sufficient amount of water at the early developmental growth stage (Wei *et al.*, 1997).

A. annua is a determinate short day plant. The flowering of the plant is very responsive to the photoperiodic stimulus. However, the interaction between photoperiod and temperature would affect the flowering of the plant. For example, the Vietnam genotype clones have their flowering accelerated in low temperature with the photoperiod between 11 and 13 hours (Marchese *et al.*, 2002). The flower of *A. annua* is tiny, globose and only 2 to 3 mm in diameter. The greenish-yellow flowers are enclosed with numerous imbricate bracts (Figure 2.1d). They are displayed in numerous loose and dropping panicles which contain numerous central bisexual florets and marginal pistillate florets. Both flowers have synpetalous tubular corolla with the top split into five lobes bisexual florets or two to three lobes in pistillate florets respectively. The central bisexual florets could be either fertile or sterile (Ferreira and Janick, 2009).

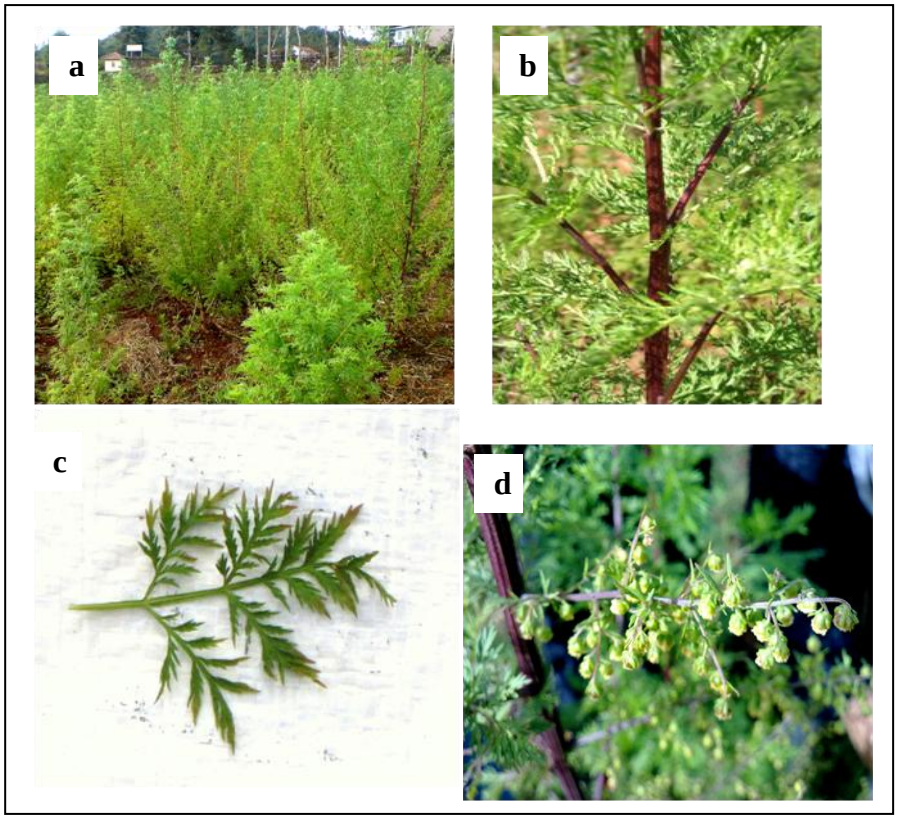

Figure 2.1 (a) Cultivation of *A. annua* at Da Lat, Vietnam; (b) the erected and ribbed stem; (c) the leaf and (d) the flower of *A. annua*

2.1.3 Trichomes

Trichomes are plant appendages that derive from epidermal cells of the plant parts such as leaves, inflorescences, stems and other plant organs. This specialized organs function as water loss controller, herbivore determent, plant defense system and production of secondary metabolites (Wagner, 1991; Dalin *et al.*, 2008). It may occur in various shapes and sizes. Several types of foliar trichomes were found in *Artemisia* plant species (Hayat *et al.*, 2009). They play important role in plant taxonomy because of their differences in shape, morphology, cell numbers and composition (Kelsey, 1984; Anthony *et al.*, 2008; Noorbakhsh *et al.*, 2008; Hayat *et al.*, 2009; Xiang *et al.*, 2009). However, there were only two main types of trichomes found in *A. annua*, the non-glandular filamentous T-shaped trichomes and glandular secretory trichomes. There are abundant T-shaped trichomes found on the leaf surface, stem and inflorescences of *A. annua*. The functions of this trichome are water absorption, seed dispersal and defense against herbivore (Ferreira and Janick, 1995; Wagner *et al.*, 2004).

Glandular secretory trichomes found in *A. annua* are mainly biseriate capitates trichomes. It is the major sites for biosynthesis, accumulation and secretion of plant secondary metabolites which usually involved in plant defense against insect predation (Duke and Paul, 1993; Duke, 1994; Ranger and Hower, 2001). Besides, it is the major part which contributes the flavor and aromatic smell of the plants. This trichome is important in *A. annua* because it stores and biosynthesizes artemisinin, an important antimalarial compound. It is mainly located on the leaves and inflorescences of *A. annua* plant. Younger leaves produce more glandular trichomes compare to older leaves. Therefore, more artemisinin is obtained from the younger leaves (Ferreira and Janick, 1996; Arsenault *et al.*, 2010).

The capitates glandular trichomes are made up of 10 cells stack in pairs with five cells on each side. The four lower cells function as a stalk to support the six upper cells. All the six upper cells bound with secretory cavity which plays the role in filling the apical subcuticular space with secondary metabolites. The earliest stage of glandular trichome formation is the one-cell stage, in which a single epidermal cell enlarges to protrude above the leaf surface. The glands are covered by the leaf primodia during the early stage of development. The ten cells structure is formed from this single epidermal cell after series of cell division (Duke and Paul, 1993).

The non-glandular filamentous T-shaped trichome is made up of five cells which present in basal bracts and pedicel of the capitulum. The top of the T-shaped trichome is formed by a greatly elongated cell (Duke and Paul, 1993; Ferreira and Janick, 1995). The arrangement of the trichomes is different on the adaxial and abaxial of the leaf. On the surface of the leaf, the T-shape and capitate trichomes are arranged in two rows along either side of the midrib. But, for the abaxial surface, the glandular trichomes are randomly scattered on the surface (Duke and Paul, 1993).

Although the production of artemisinin was found to be correlated with the number of glandular trichomes developed (Kapoor *et al.*, 2007; Arsenault *et al.*, 2010; Graham *et al.*, 2010), but the amount of artemisinin store in the trichomes varies with time. When the glandular trichomes mature, the surrounding cells senesce and stop secrete the artemisinin into the central cavity (Werker, 2000; Lommen *et al.*, 2006). For *A. annua*, the subcuticular sacs are broken in most of the mature glandular trichomes. This is because *A. annua* released artemisinin by rupturing the gland (Duke and Paul, 1993). It was also found that the density of glandular trichomes was highest when the leaves grow up to its maximum size and decrease rapidly after reaching its maximum size. This also indicated that the glandular trichomes ruptured

during plant development (Shanker *et al.*, 1999; Lommen *et al.*, 2006; Arsenault *et al.*, 2010). This finding also suggested that the aromatic smell of *A. annua* produced due to the rupturing of the glandular trichomes.

2.2 Chemical constituents of *Artemisia annua*

A. annua is an important plant source used in the treatment of malaria. Identification of the active compounds in the *A. annua* plant was carried out by several researchers with the intention to isolate the active compound that react against malarial parasites (Bhakuni *et al.*, 2002; Chen *et al.*, 2008; Muzemil, 2008). Approximately six hundred secondary metabolites were identified from *A. annua* plants. Most of these phytochemical compounds are dominated by terpenoids, flavanoids, coumarins and shikimate metabolites (Brown, 2010). The unique compound found in this plant is artemisinin (sesquiterpene lactone) which is difficult to detect in other species of *Artemisia* genus (Klayman *et al.*, 1984; Balachandran *et al.*, 1987; Erdemoglu *et al.*, 2007). However, the phytochemistry in this plant is variable between the cultivated strains and genotype. For example, the Chinese varieties of *A. annua* contain predominately artemisia ketone while the Vietnamese varieties are dominated with camphor and germacrene D (Woerdenbag *et al.*, 1993).

2.2.1 Artemisinin

Artemisinin is an endoperoxide sesquiterpene lactone, an active compound against malaria. This anti-malarial compound was successfully extracted from *A. annua* and introduced to the rest of the world since 1979 as reviewed by Klayman

(1985). The chemical structure of artemisinin (Figure 2.2) was identified with empirical formula of $C_{15}H_{22}O_5$ and molecular weight of 282.1742 g/mol (Klayman, 1985; Lin *et al.*, 1985; Luo and Shen, 1987).

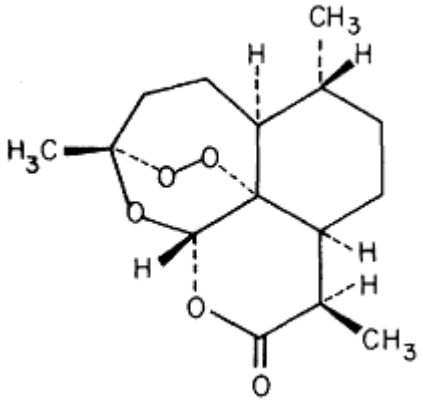

Figure 2.2 Chemical structure of the artemisinin, an endoperoxide bridge situated within the backbone of the sesquiterpene lactone (Klayman, 1985)

Artemisinin is a unique secondary metabolite found in *A. annua*. It is an odourless and non-volatile compound. It is white crystallized powder with the melting point at about 156 ℃. Further increasing the temperature to about 190 ℃ would cause the decomposition of the compound (Lin *et al.*, 1985). Artemisinin is poorly dissolved in water and oil. It is also easily decomposed in protic solvents which contain dissociable H^+ ion such as methanol and acetic acids. This solvent would cause the opening of the lactone ring structure. Reduction of the artemisinin to other derivatives could also occur in acid and alkali solutions due to hydrogenation of the endoperoxide structure (Liu *et al.*, 1979; Zeng *et al.*, 1983; Zhou and Wen, 1984). However, this compound is soluble and stabilized in most of the aprotic solvents (Klayman, 1985). Artemisinin is also light sensitive and decomposed when long exposure to the light (White, 2002).

Currently, the demand of artemisinin throughout the world is high due to its antimalarial properties especially against chloroquine resistant strain *Plasmodium falciparum*. Artemisinin showed rapid parasites clearance in blood and disappearance of fever as compared to chloroquine (Tu, 2011). It is toxic to the parasites even apply in very low concentration. Artemisinin not only affect the mature parasites at the sexual stages, but also effective against parasites at the asexual stages (Terkuile *et al.*, 1993). It also caused the structural change of parasites in the erythrocytes stage (Dhingra *et al.*, 1999). However, the mechanism of artemisinin action against the malarial parasites remains uncertain but the endoperoxide bridge is essential for antimalarial properties. This endoperoxide structure would generate free radicals when exposed to unbound iron or heme. The growth of malaria parasites in the red blood cells would cause the accumulation of excess iron that would activate the activities of artemisinin (Krishna *et al.*, 2004). Integration of this endoperoxide structure would produce the free radicals that are used to attack and destroy the parasites membrane system (Hien and White, 1993).

In contrast with other antimalarial drugs, artemisinin provides broad specificity of antimalarial action. It reacts against broader age ranges of the parasites (Terkuile *et al.*, 1993). The therapeutic reactions of artemisinin consist of killing young circulating ring-stage malarial parasites (trophozoite) in the blood stream and reducing the number of mature parasites that sequester and block the blood capillaries and venules (Udomsangpetch, 1996; Angus *et al.*, 1997; White, 1997). This showed that the efficiency of artemisinin in rapid clearance of malaria parasites and in treatment of the severe malaria as compared to other antimalarial drug which only kill parasites in the mature stage. Removing the malaria parasites at the gametocytes stage (inactive young stage) would reduce the number of mature

parasite (infectious stage) release into the blood stream. Therefore, the transmission potential to other parts of the body would be reduced (White, 2008).

In addition to malarial parasites, artemisinin has been reported to act against other parasites such as Leishmania (Sen *et al.*, 2007) and Toxoplasma (Dunay *et al.*, 2009). Artemisinin also found to have cytotoxicity effect on tumour cells in the present of ferrous iron. The anti-cancer activities were found in the inhibition of cell growth in several neoplastic cell lines (Xu *et al.*, 2007). High accumulation of iron in the cancer cells is the factors that made it more sensitive towards free radicals attacked from artemisinin as compared to the normal cells (Lai and Singh, 1995; Lai *et al.*, 2005). Destruction of breast cancer cells was observed with the treatment of artemisinin in the *in vitro* system (Singh and Lai, 2001). Artemisinin also showed antiviral and anti-inflammatory activities. Strong activities of artemisinin were observed against bovine viral diarrhoea virus of the Flaviviridae family (Romero *et al.*, 2006). It was also found to have certain inhibition activity against human cytomegalovirus, hepatitis B virus and hepatitis C virus (Efferth *et al.*, 2008).

2.2.2 Derivatives of Artemisinin

Although artemisinin is very efficient against malarial parasites at very low concentration and excellent tolerability in human body, mono-therapy by using artemisnin itself is practically inefficient. Poor solubility of artemisinin in water and oil made it unsuitable for oral consumption and poor absorption by gastro-intestinal tract. Recrudescence of the parasites was observed after the treatment due to its short-plasma half life. At least seven days of treatment is required to prevent recrudescence if using artemisinin mono-therapy (Price *et al.*, 1999; Chaturvedi *et al.*,

2010). Therefore, the used of artemisinin and its derivatives as a combination therapy are normally employed in the treatment of malaria (White, 2008).

Several derivatives of artemisinin were discovered such as dihydroartemisinin, artesunate, artemether and arteether. These derivatives provide similar effect in the treatment of the malarial parasites but more efficient in term of chemical and physical stability of the molecular structure. Large scale derivatization of artemisinin was not preferable. However, artemisinin is easily reduced to more stabilized compound which is dihydroartemisinin by using sodium borohydride. This semi-derivative compound was used in the preparation of the water soluble artesunate and oil soluble artemether and arteether (Hien and White, 1993).

Dihydroartmisinin and other derivatives such as artesunate, artmether and arteether are more potent drugs compare to its parent compound artemisinin. These derivatives were 20 to 100 times more active than the parent compound (Alin *et al.*, 1990). However, artemisinin and its derivatives such as artemether, arteether and artesunate were found to be rapidly converted to dihydroartemisinin *in vivo* (Lee and Hufford, 1990). But, the derivatives have higher half life compare to its parent compound so their elimination from the plasma cells is slower. Therefore, the derivatives are more efficient in parasites clearance.

2.2.3 Artemisinin Production in Plants

Since the artemisinin was critical in the treatment of severe malaria, the demanding of this compound becomes higher especially to countries where malaria is endemic. Till now, artemisinin production is unable to meet the world demand (Shretta and Yadav, 2012). The full chemical synthesis of the artemisinin has been

achieved but is too complex and result in high cost which is not suitable and uneconomical for commercialization (Yadav *et al.*, 2003). Hence, large scale production of artemisinin has to depend on the natural synthesis in the plant. However, the yields of artemisinin from field grown plants are subjected to environmental and geographical conditions such as temperature, humidity, soil type and harvesting time (Wright, 2002; Zhang *et al.*, 2008; Thu *et al.*, 2011; Omer *et al.*, 2013). Due to these limitations, harvesting the artemisinin from the wild plants is uncertain and not sustainable. Therefore, cultivation of *A. annua* is still needed. China and Vietnam are the major producers of artemisinin derive from plant cultivation (Ferreira, 2005; White, 2008).

As mention earlier, artemisinin is sequestered in the glandular trichomes which are found on the leave surfaces of the plant. Therefore, distribution of artemisinin in the plant parts is depended on the present of the glandular trichomes (Duke and Paul, 1993). Artemsinin can also be detected in different plant organs such as leaves, stems and flowers but no detection in pollens or roots (Klayman, 1993; Ferreira *et al.*, 1995). This is because glandular trichomes are not found in the roots of the field grown plants. Nevertheless, the accumulation of artemisinin concentration in the plant varies at different stage of the vegetative growth and diverse in various plant parts (Laughlin *et al.*, 2002). Therefore, it is important to determine accurate harvesting time when the accumulation of artemisinin is at the highest and to ensure optimum production.

An evaluation of the artemisinin content in the leaves and inflorescences at different developmental stage showed that the content of artemisinin was highest during full bloom as compared to pre and post-flowering (Yeboah *et al.*, 2011). Artemisinin content at pre and post flowering stage is 50 % less than during full

bloom. The content of artemisinin in the inflorescences was found to be higher than the leaves at pre-flowering stage but during blooming stage the content in the leave was higher (Baraldi *et al.,* 2008). However, there were some contradicting results stated that the accumulation of artemisinin was the highest at the vegetative developmental stage before flowering (Acton and Klayman, 1985; ElSoly *et al.,* 1990; Woerdenbag *et al.,* 1991). Gupta *et al.* (2002) reported that the artemisinin content was highest in the leaves at pre-flowering vegetative stage. The content of Artemisinin in the leave was as high as 1.1%. Roots were devoid of artemisinin at any development stage of the plant.

Besides this, artemisinin content in the leave also vary at different position on the plant. The content was relatively higher in the leave at the segment approaching the top of the plant. The leaves of different order of branching also affect in variation of artemisinin content. Fine leave from higher branching order contained higher artemisinin content (Gupta *et al.,* 2002). However, for commercialization or large scale production, whole aerial parts of the plant would be harvested rather than selection based on leave position.

Kumar *et al.* (2004) reported that extending harvesting time of plant would increase the content of the artemisinin as its yield was proportional to the growing age. Higher artemisinin content was found in plants that allowed to grow in the field for longer period as compared to those that harvested earlier (Ram *et al.,* 1997). Results showed that increasing growing age from four to seven months after transplanting increase the content of artemisinin by 400 % (Damtew *et al.,* 2011). Extending growing time also allowed the plant to produce more biomass, hence, increase yield of artemisinin per plant. However, senescence of older leaves cause the lost of artemisinin if the harvesting period was extended (Gupta *et al.,* 2002).

Climate and geographical conditions could affect the production of artemisinin in the plant. The artemisinin content in the plant is higher when planting in subtropical region. The climate at the subtropical region provides suitable growing condition for the plant. Temperature between 13.9 ℃ and 22 ℃, sunshine duration between 835 and 1507 hours and rainfalls between 814 and 1518 mm are the best conditions for production of artemisinin (Zhang *et al.*, 2011). Planting of *A. annua* at tropical region are unsuitable for artemisinin production as flowering would be induced at early developmental stage when the plant is still very small result in low plant biomass (Klayman, 1993). Drought is also one of the factors that induced early flowering and affect the artemisinin level in the plant (Charles *et al.*, 1993; Marchese *et al.*, 2010; Wilcox *et al.*, 2011). Variation in artemisinin production also observed in plants harvested from different altitude. Plant grown in higher altitude at about 1500 to 2000 m from sea level produce higher amount of artemisinin. Approximately 1.1 % artemisinin from dried biomass could be obtained from field grown plant if the plants grow at optimum condition (Thu *et al.*, 2011; Larson *et al.*, 2013).

Genotype and geographical origin of the plant selected for planting could also affect the yield of artemisinin (Marchese *et al.*, 2002). Selection of high productive line for mass production is crucial to ensure optimum yield of artemisinin. Crosses between high yielding genotype should produce progenies with high artemisinin content. A selected hybrid line was found to produce 1 to 2 % of artemisinin (Delabays *et al.*, 2001; Ferreira *et al.*, 2005). For planting in tropical climate region, continuous selection of high producing lines is required to isolate the lines that can produce sufficient biomass and artemisinin for further cultivation. The important criteria in the selection of productive lines are based not only on the artemisinin production, it is also important to select plant with high leaf to stem dry mater ratio,

tall and late flowering plant (Ferreira *et al.*, 2005). Selection of late flowering plant is to enable the plant to reach the optimum vegetative growth before entering reproductive stage. This would increase biomass production of the plant especially at tropical region where the photoperiod is shorter.

Deficit of quality seeds is also a constraint for artemisinin production. Harvesting the plant before flowering is the major reason caused deficiency of seeds availability (Acton and Klayman, 1985; Gupta *et al.*, 2002). In addition, seed generated plants produce wide variation in artemisinin content (Ferreira *et al.*, 1997). It can range from 0.01 % (Trigg, 1989) to 1.5 % (Debrunner *et al.*, 1996). With all these constrains *in vitro* culture techniques can be used as the alternative tools for the production of artemisinin using the selected elite clones.

2.3 *In vitro* Culture Technology

2.3.1 *In vitro* Production of Secondary Metabolites

In vitro plant culture technology refers to the growth of plant cells, tissues or organs derived from the mother plant under controlled condition on artificial medium. The artificial medium normally contains the basic nutrients for plant growth. The *in vitro* culture techniques can be used for plant propagation. It can also be used as a tool for in the production of useful plant secondary metabolites (George and Sherrington, 1984). The discovery of plant capability to produce secondary metabolites under *in vitro* condition gave an impact to pharmaceutical industry especially in the production of active ingredient from plant (Vijaya *et al.*, 2010). Pharmaceutical compounds such as alkaloids, terpenoids, steroids, saponins,

phenolics, flavanoids, and amino acids were successfully produced via *in vitro* culture system (Abdin and Kamaluddin, 2006; Debnath *et al.*, 2006; Anand, 2010).

Studies have been proven that production of bioactive compounds from field cultivation could be replaced by *in vitro* culture as the compounds produced *in vitro* are parallel to the compounds produced from the whole plants. Secondary metabolites can continuously produce throughout the year using the *in vitro* culture technology because there are no seasonal or weather constraints. *In vitro* culture of plant cells can be established routinely from explants such as leaves, stems, roots and other plant parts for cell proliferation and production of secondary metabolites (Hussain *et al.*, 2012). The production of the active compounds is also reliable and predictable as compare to field grown plants. Isolation of the compounds is easier than from the whole plant which is more complex. Besides, interfering compounds occur in field grown plant could be avoided by using *in vitro* culture technique (Karuppusamy, 2009; Anand, 2010).

In vitro production of secondary metabolites can be carried out via two approaches that is differentiated culture or undifferentiated culture. In the differentiated culture, plant organ such as leave, stem or root were generated *in vitro* as certain products of interest were only produced in specialized tissue in the plant (Dornenburg and Knorr, 1997). For example production of saponin from *Panax ginseng* is only found in the roots (Yendo *et al.*, 2010). For undifferentiated culture such as callus or cell culture, differentiation on specific organ are not needed, the culture only undergoes cell multiplication to gain biomass for the production of bioactive compounds (Siashar *et al.*, 2011).

As *in vitro* culture technology would give rise to the progeny which is identical from the mother plant, initial selection for production and multiplication is important (George *et al.*, 2008). Selection of high producing strains can lead to sustainable and enhancement in the production of secondary metabolites. Besides, optimization of medium and microenvironment also require to ensure optimum production of secondary metabolites (DiCosmo and Misawa, 1995).

Since production of artemisinin from field grown plant are inconsistent and influenced by several exogenous factors especially environmental condition and geographical location, *in vitro* culture technology can provide an alternate for artemisinin production from *A. annua* under control environment (Rao and Ravishankar, 2002). Furthermore, *in vitro* technology enables production of genetically uniform plants which overcome the concern of genetic variation in the field grown plants which derived from seeds. Moreover, a large number of plants can be produced within limited time and space using the *in vitro* culture technology (Allan, 1981; Gurel, 2009).

2.3.2 Callus Culture

Callus is an unorganized tissue which can be derived from a wide variety of plant parts such as leaves, stems, roots or other specific cell types under vigorous cell division (George and Sherrington, 1984). Callus occurs as a mass of undifferentiated cells under *in vitro* condition which may proliferate indefinitely under routine transferring or subculture to a fresh medium (Thorpe, 1982; Allan, 1991; Caponetti, 1996).

In *in vitro* culture system, initiation of callus begins with the swelling at the cutting edges of the explants when culture on a suitable culture medium which usually consists of plant growth regulators (George and Sherrington, 1984). Callus initiation could be observed within four to five weeks after culturing of the explants onto a solid or semi-solid medium. The callus can remain in contact with the explants until sufficient amount is formed. This is because plant cells need to have minimum inoculation density for growing. Further growth would be inhibited if the amount of callus transferred does not meet the minimum inoculation amount (Dixon, 1985; Torres, 1988; Purohit, 2013). Although callus is derived from different tissue that might have different minimum inoculation density, the amount of callus to be transferred at every subculture cycle should be relatively constant at regular intervals of three to five weeks. This is to maintain the stability of the callus cell lines (Allan, 1991).

Callus culture is heterogeneous culture and this characteristic can be observed as it comes in different colour, poses different morphology, growth and metabolism. Even the visually observed uniform callus was found to have different metabolite production capability (Allan, 1991; Zhong and Yue, 2005). Growth pattern of the callus is inconsistent as the nutrients gradient occurred during the growing of the callus. This is because the portions of callus in contact with the medium are different. This also indicated that there are no identical calluses although placing onto the same medium. However, variation of the calluses might become less through routine subculture and the consistency could be attained (Chan *et al.*, 2008; Christine *et al.*, 2011; Karthic and Seshadri, 2012).

Since the variation and heterogeneity occur in callus culture, callus selection has to be carried out for obtaining consistent and productive lines. Selection should

start at the first transferring process when degree of variation is the greatest. Therefore, a number of callus cell lines could be obtained (Allan, 1991). This is an important step for the *in vitro* production of bioactive compounds because different callus cell lines might produce different amount of desired compounds. It also protects the loss of genetic material during callus initiation (Mulabagal and Tsay, 2004). During the subculturing process, certain portions of the callus might be discarded. However, if the portion of callus transferred reached the minimum inoculation density, the cell within a tissue can generate a sector that retains their special characteristic (Amasino and Miller, 1982; Sussex, 2008). Therefore, callus culture can provide an alternate way for germplasm conservation as it can be maintained in long periods (Alejandrina and Hector, 2012).

Callus formation from plant tissue is determined by the level of plant growth regulator. It is a major factor that controls the induction of callus formation (George *et al.*, 2008). Concentration of plant growth regulators needed is depended on the plant species and also the type of explants used (Anna *et al.*, 2013; Ujjwala *et al.*, 2013). Auxins and cytokinins are the important plant growth regulators which involve in the formation of callus. The major roles of auxins are in the elongation of cell, root formation and stimulation of embryogenesis while cytokinins involved in cell division and shoot formation (George and Sherrington, 1984). Therefore, intragenous and exogenous balance of auxins and cytokinins are important in the production of callus (Allan, 1991; George *et al.*, 2008).

Callus culture was successfully established for *A. annua* from different types of explants either with auxins alone or combination of auxins and cytokinins (Wu *et al.*, 2007; Shi *et al.*, 2008; Bartarya *et al.*, 2008; Ganesan and Paulsamy, 2011; Jamaleddin *et al.*, 2011). Leaf explants were used in *A. annua* callus production. The