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Investigation on the effect of subculture frequency and inoculum size on the artemisinin content in a cell suspension culture of *Artemisia annua* L.

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Abstract

Artemisinin, an endoperoxide sesquiterpene lactone, has been proven to be effective for the treatment of both drug resistant and cerebral malaria parasites. It is isolated mainly from the aerial parts of *Artemisia annua* plants but the production is not sufficient for the current market demand. Chemical synthesis of artemisinin was found to be difficult and not economically viable. Therefore, the enhanced production of artemisinin via the *in vitro* cell culture technique is highly desirable. Friable callus is required for the preparation of the cell suspension culture. The callus cultures of *A. annua* were initiated from the leaf explants. Light green friable calli at the fourth subculture cycle were chosen as the best callus age to initiate cell suspension culture. The *A. annua* cells could be maintained with a growth index of between 10 and 18 for 33 subculture cycles with 16 days interval per cycle. The highest artemisinin content was detected in cultures with initial inoculum of 0.50 g after 12 days of culture in the callus induction medium.

Keywords: antimalarial; callus age; callus induction; cell culture; growth index; secondary metabolite.
Abbreviations: BAP- 6-benzylaminopurine; MS- Murashige and Skoog; NAA-naphthaleneacetic acid.

Introduction

A few species of *Artemisia* from the family Asteraceae, namely, *Artemisia annua* (qinghao), *A. apiacea*, *A. lanceolata* and *A. scoparia*, have been reported to contain artemisinin, a potent antimalarial drug (Singh and Sarin, 2010; Willcox et al., 2004). However, most studies focused on *A. annua* due to its higher concentrations of artemisinin (Hsu, 2006). Currently, there are no substitutes for artemisinin in the fight against malaria inasmuch as it can be used to treat severe malaria that has developed a resistance to previous antimalarials. It can be administered by several routes, has more rapid action and is able to attack a broader age range of protozoan *Plasmodium* parasites (Dhingra et al., 1999; Dondorp et al., 2010; Krishna et al., 2004; White, 2004). Besides its recognized antimalarial property, artemisinin from *A. annua* was found to be an effective anticancer (Nakase et al., 2008) and anti-inflammatory agent (Wang et al., 2007). One of the advantages of artemisinin is that it eliminates cancer cells by inducing apoptosis while, at the same time, it is non-toxic to the normal cells (Nakase et al., 2008). The commercial production of artemisinin has been hindered because of the low artemisinin content in *A. annua* plants and the non-availability of an economically feasible synthetic protocol (Weathers et al., 2006). The cell culture technique is an excellent method to study the biosynthesis of secondary metabolites in plants (Ramachandra Rao and Ravishankar, 2002; Zhong et al., 2001). Plant cell cultures are more favourable in comparison to cultures in a solid form medium because cells are uniformly bathed in the liquid culture medium, which results in higher tissue contact with the nutrients in the medium (Hippolyte et al., 1992; Soomro and Memon, 2007). This technique also provides a better alternative for plants that are difficult to cultivate and/or have a long cultivation period (Hippolyte et al., 1992). The rapid and short growth cycles of plant cells in *in vitro* culture makes large-scale production of novel secondary metabolites possible. Also, the production and accumulation of secondary metabolites in the plant cell cultures can be enhanced with the manipulation of chemical and physical microenvironments and the selection of high yielding cell clones (Mulabagal and Tsay, 2004). Attempts have been made to produce artemisinin via the cell culture technique on other varieties of *A. annua* of different origin (Baldi and Dixit, 2008; Chan et al., 2010). However, there is no report on the production of artemisinin from the callus or cell cultures initiated from *A. annua* originating from Vietnam. Initial inoculum density is a key factor in establishing cell suspension cultures of any plant species because cells cannot resume or even stop their active growth after transfer if the initial inoculum is below the critical cell density (Franklin and Dixon, 1994; Street, 1977). This paper reports the growth kinetics, effect of subculture frequencies of the *A. annua* on callus and cell cultures, effect of age of callus on the establishment of cell culture, and effect of inoculum size on artemisinin content in the cell suspension culture of *A. annua*.

Results

Establishment of A. annua callus culture

The excised leaf pieces enlarged and swelled to two to three times compared with the original size when in contact with the callus induction medium. Callus formation first took place at the cutting edges of the leaf pieces after

approximately 14 days of culture. Then, a mass of cells gradually grew over the leaf pieces within 35 days. Callus was found to easily proliferate in this medium, which was optimized for different origins of *A. annua*. A reddish purple or pink pigment was observed at the primary callus and appeared on the upper surface of the callus cultures after subculture. The calli adjacent to the culture medium were hard, compact and green in colour, whereas, the upper part of the calli was soft, amorphous, and yellowish green and light green in colour.

Growth kinetics and effect of subculture frequency on growth of A. annua callus culture

A. annua callus cultures showed sigmoid growth pattern. The growth index and dry weight of callus increased gradually from the first week of culture until the fifth week and fourth week, respectively (Fig 1). Reddish purple callus on the surface became dark from the fifth week onwards. Yellowish green, light green and green callus turned to brown colour in the sixth week. All calli became dark and growth ceased at the end of the seventh week culture period. Also, browning of the culture medium was observed at the seventh week. Therefore, calli were maintained with subcultures at four-week intervals in a fresh medium to produce stable callus cultures and to maintain callus viability. As for the effect of subculture frequency on callus cultures, a short transient period was noted during the first and second subculture of *A. annua* callus cultures as the cultures showed high variations in both growth index and dry weight obtained (Fig 2). The growth index and dry weights of *A. annua* calli were found to increase proportionally with the number of subcultures and became stable after six subcultures with a growth index of ± 25.7. A check was carried out from the 18th subculture onwards and it was noticed that the growth index of callus increased to 34.9. Production of *A. annua* callus peaked at the 20th subculture with a growth index of 41.6, which then decreased to ± 31.9 at further subsequent subcultures. The appearance of the reddish purple calli became rare and reduced to almost nil with the number of subcultures carried out (Fig 3).

Establishment of A. annua cell suspension culture

The callus produced before four times of subculture was found to be unsuitable for the initiation of *A. annua* cell suspension cultures (Fig 4). Cell suspension cultures initiated from three subcultured calli initially consisted of green cellular clumps, and, subsequently, most of them turned brown due to stress in the liquid form medium. The cells that remained on the filter paper after the liquid medium was removed with the air suction pump were sticky. Also, the liquid medium of the cultures appeared cloudy and brownish indicating cell death, and, hence, not practical to subculture. Therefore, the callus at the next subculture was chosen for initiating cell suspensions. Finally, cell suspension cultures were successfully initiated from the callus at the fourth subculture and onwards. The growth index of cell cultures induced from the fourth subculture callus increased gradually with subsequent subcultures. Cells were fluffy and lush green in colour.

Growth kinetics of A. annua cell culture and effect of subculture frequency

All four sets of cultures with different initial inoculums showed sigmoid growth curves when the fresh weight and

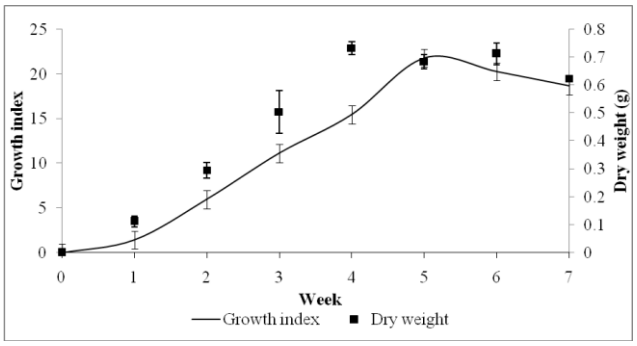


Fig 1. Growth kinetics of *A. annua* callus cultures based on growth index and dry weight in MS medium supplemented with 0.5 mg/L BAP, 0.5 mg/L NAA and 0.5 g/L casein hydrolysate over a culture period of seven weeks. Bars represent mean ± standard error of six replications.

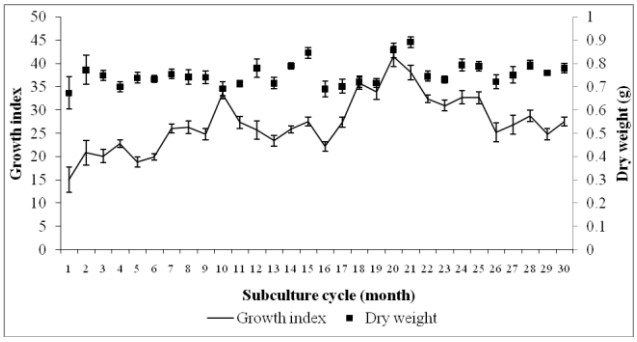


Fig 2. Subculture effect on growth index and dry weight of *A. annua* callus. Bars represent mean ± standard error.

dry weight were plotted against a 30-day culture period (Fig 5). The highest growth indexes for cultures with initial inoculums of 0.25 g, 0.50 g, 0.75 g and 1.00 g were 38.3, 19.5, 13.9 and 9.7, respectively. Interestingly, the maximum growth index was attained with an inoculum density of 0.25 g, which was the lowest inoculum size. The cultures with higher initial inoculums seemed to show limited growth when compared with cultures with lower initial inoculums, as seen by the growth indexes. Therefore, a higher initial inoculum did not produce greater cell biomass. Based on fresh weight, 0.25 g initial inoculum cultures had prolonged lag phase (9 days) compared to the other three sets of cultures (6 days). After the lag phase, the cells of all four sets of cultures were dividing and grew vigorously entering their exponential phase. The growth rate of cells continued to increase exponentially until it reached a stationary phase where the growth of cells ceased (Fig 5a). For all cell cultures, browning was observed and growth decreased gradually when cultures entered the stationary phase. However, the dry weight, at day 18, for cultures with four different initial inoculums produced approximately the same amount of dry cell mass (Fig 5b). Initial inoculums of 0.25 g cultures reached the highest growth rate later than the other three sets of cultures, which might increase the contamination rate as the cultures were exposed to more contaminants during the prolonged culture period. An inoculum size of 0.50 g was found to be the most suitable initial inoculum. This was because its growth index was higher than cultures with 0.75 g and 1.00 g initial inoculums even though they

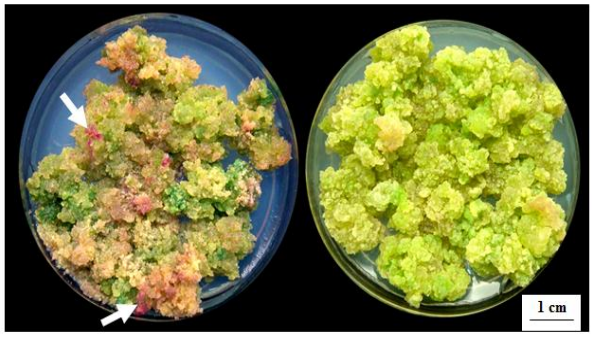


Fig 3. A hint of reddish purple calli (arrow) appeared on upper layer of callus produced during the first few subculture of *A. annua* callus (left). After more subculture were carried out, the appearance of the reddish purple calli were reduced to almost none (right).

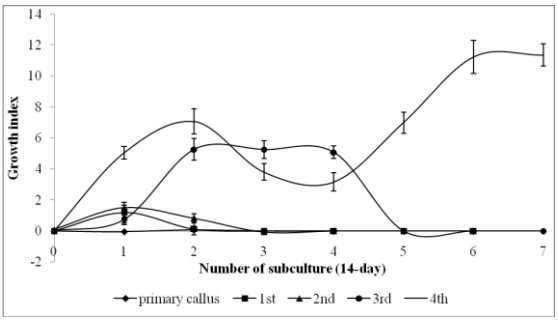
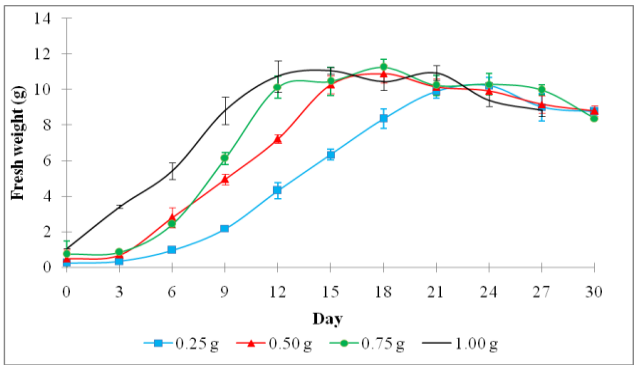


Fig 4. Influence of callus age on the establishment of cell suspension cultures of *A. annua*.

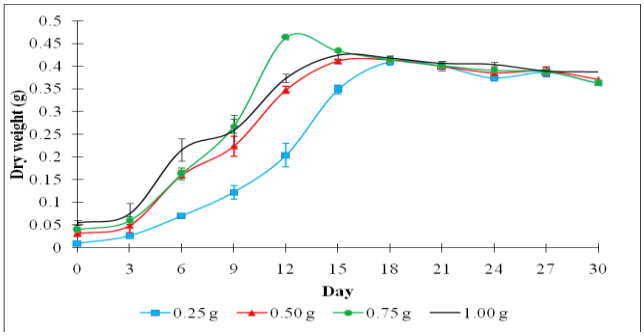
achieved maximum growth by the same day (18th day) if based on fresh weight or dry weight. Therefore, 0.50 g of *A. annua* cells was chosen to transfer into a fresh medium at a 16-day interval, before the culture entering stationary phase, to gain optimum growth of cells. The growth of *A. annua* cells was found to become stable from the 6th subculture until the 8th subculture (Fig 6). Therefore, the growth kinetics of the *A. annua* cell culture was carried out using cells from the 7th and 8th subculture. Initially, *A. annua* cells comprised a mixture of fine cells and small cell aggregates. However, after subsequent sieving through an 850 µm sieve, small cell aggregates were reduced and consisted of fine cells only. Although clumping of cells was observed occasionally during subculture, these were easily broken mechanically with a spatula. Thus, sieving of cells was no longer required to maintain cell cultures consisting of fine cells. Over the 33 subculture cycles, *A. annua* cells were well maintained in callus induction medium and retained their lush green colour (Fig 7).

Extraction of artemisinin from the cell cultures

The artemisinin content in cultures with 0.25 g initial inoculum decreased gradually and contained no artemisinin at all from the 24th day until the 30th day. Whereas the content of artemisinin in 0.50 g initial inoculum cultures fluctuated within the 30-day culture period. Even though the highest growth rate of 0.25 g initial inoculum cultures was about twice as much compared to the highest growth rate of 0.50 g initial inoculum cultures, no trace of artemisinin was detected. No artemisinin was detected in cultures with 0.75 g



(A)



(B)

Fig 5. Growth kinetics of *A. annua* cell suspension cultures based on fresh weight **(a)** and dry weight **(b)** in MS medium supplemented with 0.5 mg/L BAP, 0.5 mg/L NAA and 0.5 g/L casein hydrolysate with four different initial inocula (0.25, 0.50, 0.75 and 1.00 g) over a culture period of 30 days. Data were collected from six replications. Bars represent mean ± standard error.

and 1.00 g cultures, however, 0.02 % of artemisinin was detected at three-day-old culture of 1.00 g initial inoculums (Fig 8). It was further confirmed that 0.50 g was the optimum initial inoculum, as it produced the highest artemisinin (0.06%).

Discussions

Enlargement of leaf explants during callus induction was also observed in other studies regardless of the family or species of explants (Jawahar et al., 2008; Komal, 2011; Preethi et al., 2011; Rout et al., 2009). Leaves of rooted *in vitro* plantlets of *A. annua* were used to induce callus because the presence of roots was proven to enhance the production of artemisinin in shoots (Ferreira and Janick, 1996). The appearance of anthocyanin like reddish purple pigment on *A. annua* callus in this study was also observed in gall calli of *A. annua* (Ghosh et al., 1997). The rate of growth of *A. annua* callus and cell suspension was determined because it was essential in preserving their ability to increase biomass and continue to multiply (Robledo-Paz et al., 2005). Both callus and cell cultures of *A. annua* showed a sigmoid growth pattern, which started with the lag phase, followed by the exponential phase and ended with the stationary phase. This typical growth pattern was in agreement with many other plant callus and

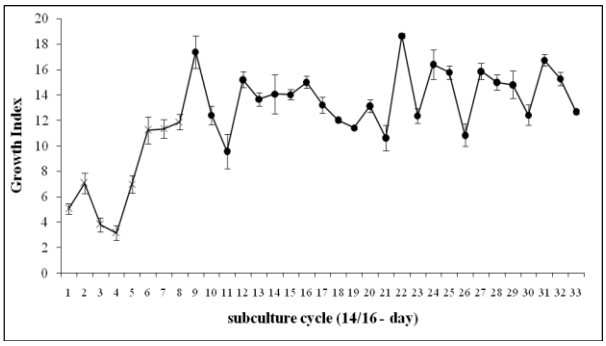


Fig 6. Effect of subculture frequency on *A. annua* cell suspension culture. First eight subcultures were carried out with 14-day interval whereas 8th subcultures onwards were carried out with 16-day interval. Bars represent mean ± standard error of seven replications.

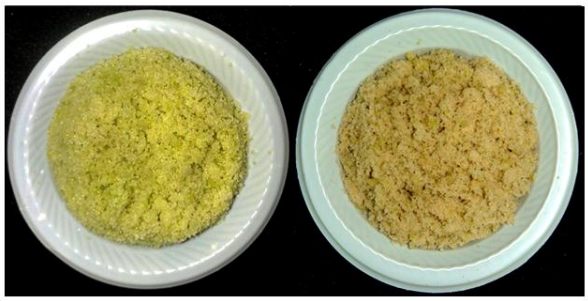


Fig 7. Healthy lush green cells at initial subculture cycles (left) turned pale light green after more subculture was carried out (right). Healthy lush green cells were occasionally obtained in the later subculture cycle.

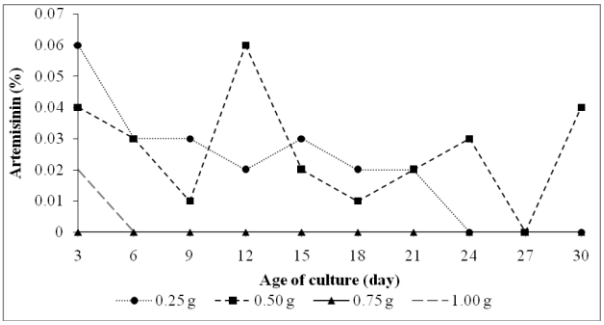


Fig 8. Artemisinin (%) detected in four different sets of cultures with 0.25 g, 0.50 g, 0.75 g and 1.00 g of initial inoculum over 30-day culture duration.

cell cultures (Karam et al., 2003; Kolewe et al., 2010; Sujanya et al., 2008). *A. annua* callus and cell growth slowed down when entering the stationary phase. Hence, subcultures have to be carried out before the cultures enter the stationary phase in order to maintain the cell lines for prolonged durations and in a healthy condition. For the duration of the exponential phase, acceleration of cell growth and proliferation was evident because the highest number of cells was in the metaphase (Smith, 2000). Exhaustion of nutrients in the culture medium, drying of solid media or concentration by evaporation of liquid media, accumulation of toxic by-products, tissue metabolites or dead cells, and oxygen depletion are the growth-limiting factors in *in vitro* cultures during the stationary phase (Karam et al., 2003; Neumann et al., 2009a; Smith, 2000; Yeoman and Macleod, 1977). Friable calli were used and inoculated into a liquid medium because the calli broke easily in an agitated liquid medium and formed more homogenous suspension cultures. Newly induced callus was not found suitable for inoculating into a liquid medium for the establishment of cell suspension culture; this was because the newly initiated calli were still adapting to the solid form medium. The transferring of newly initiated callus into the liquid form medium was detrimental to them. The growth index of *A. annua* cell cultures was inversely proportional to the initial inoculum. This was because exhaustion of oxygen or depletion of nutrients in the culture medium occurred at the beginning of the culture period due to the excess of inoculum (Blackhall et al., 1999; Contin et al., 1998). Higher initial inoculum size cultures may have suffered from a greater limitation of oxygen, thus resulting in lower or nil artemisinin production and accumulation (Contin et al., 1998). Leakage of growth factors from the cells to the surrounding medium was more significant if the number of cells transferred per volume of medium was lower (Carvalho and Curtis, 1999; Street, 1977). Cell suspension cultures of *A. annua* were a mixture of free single cells and various sizes of aggregate. The study has shown that single-cell cultures are unattainable because single cells in a liquid medium tend to aggregate shortly after initiation of growth (Neumann et al., 2009b). With the use of a suitable initial inoculum size, higher production and accumulation of secondary metabolites could be achieved as it has been found to affect the production of metabolites and cell biomass in many other plant cell cultures (Akalezi et al., 1999; Lee et al., 2006; Zhang et al., 2002; Zhao et al., 2001).

Materials and methods

Plant materials

Seeds of *A. annua* (Vietnam origin, clone TC2) were used to establish *in vitro* plantlets. They were maintained on MS (Murashige and Skoog, 1962) medium without any plant growth regulators, supplemented with 30 g/L sucrose, 8 g/L of agar (Algas, Chile) and placed under constant temperature of 24° ± 2°C with a light intensity of approximately 32.5 µmol m^{-2} s^{-1}. The pH of the medium was adjusted to 5.7 before autoclaving (TOMY ES-315) at 121°C for 11 minutes under 1.05 kg/cm^2 pressure. Subcultures were carried out using nodal segments and the shoot tip of the *in vitro* plantlets at eight-week intervals.

Callus culture

Leaves of *in vitro* rooted plantlets were used to induce callus. Three aseptically excised leaf pieces (≈ 1 cm x 1 cm) were cultured in glass culture vessels (250 ml) containing 40 ml of MS medium supplemented (per litre) with 0.5 mg BAP, 0.5 mg NAA, 0.5 g casein hydrolysate, 30 g sucrose and 8 g of agar to induce the formation of callus (Chan et al., 2010). The appearance of any primary callus was observed every few days.

Growth kinetics and effect of subculture on callus culture

An initiated callus (primary callus) of 0.5 g was inoculated and cultured in a medium with the same conditions as above. The growth of callus was evaluated through the callus growth index and dry weight gain. Six replications were harvested every week for seven weeks. The production of callus was

recorded and the growth index of callus was calculated and plotted against time to determine the growth pattern. The fresh and dry weights of callus were determined using a digital weighing scale. The callus dry weight was obtained by air-drying fresh callus at room temperature until a constant weight was attained. After the optimum culture duration was determined, callus (friable and/or compact) was randomly chosen to continue the subculture for the study of the effect of subculture frequency on callus production. Compact healthy callus was broken up mechanically with a spatula before inoculation. The effect of subculture frequency was determined with an initial inoculum of 0.50 g as well. The production of callus was recorded and the growth index was determined at the end of the subculture cycle starting from the primary callus (callus formed on the original explants) until the 24th subculture cycle. Seven replications were used for each subculture cycles. The morphology and colour of the callus obtained were also observed and recorded. The Growth index of callus was determined according to Godoy-Hernández and Vázquez-Flota (2006).

Cell suspension culture

Normal callus (0.5 g) at the end of each subculture period was transferred into a 100 ml Erlenmeyer flask containing 25 ml of the same medium as for the callus induction medium, but deleting agar to initiate cell suspension culture. The flasks were covered with a centre-drilled (0.5 mm in diameter) rubber stopper stuffed with cotton wool and covered with a small transparent plastic bag. The chosen calli were slightly broken up mechanically with a spatula before inoculating into the liquid medium. All cultures were placed on an orbital shaker at 120 rotations per minute and incubated under the same conditions as callus cultures. At the end of the 14-day culture interval, cells were sieved through an 850 µm pore size sterilized stainless steel sieve (Retsch), then, suction-filtered using an air suction pump to remove the old liquid medium before being inoculated into a fresh liquid medium. Cells with a diameter < 850 µm were used for subculturing. Seven replications were used for the establishment of the cell suspension cultures.

Growth kinetics and effect of subculture on cell culture

The growth kinetics and artemisinin production of *A. annua* cells were determined over a period of 30 days with four different initial inoculum sizes: 0.25, 0.50, 0.75 and 1.00 g. During the 30-day culture period, the cell fresh weights obtained of three replications were recorded every three days. The experiment was repeated twice. The cell fresh weight of each initial inoculum size was determined by harvesting the cells from the liquid medium with suction filtering using an air suction pump without filtering the cells through a sieve. The growth index of cell cultures were calculated and plotted against the culture duration. The cell dry weight was obtained as mentioned previously. After the optimum culture duration was determined, *A. annua* cells were further subcultured to study the effect of the subculture frequency on cell growth. For the effect of the subculture frequency on *A. annua* cell suspension culture, 1st – 8th subcultures were carried out with a 14-day interval (before optimization of subculture duration) whereas the 8th subcultures onwards were carried out with a 16-day interval. Data were collected from seven replications. The morphology and colour of the cell aggregates that remained on the filter paper (Whatman No.1) were also observed and recorded.

Determination of artemisinin

A. annua dried cells were powdered with a mortar and pestle. A dried cell mass of 0.1 g was weighed and ultrasonicated at 40 ^{o}C over a period of 1½ hours in 2 ml of n-hexane. The supernatant of the hexane extract was collected in a tube and 2 ml of new n-hexane (AR grade) was added in the 0.1 g *A. annua* dried cells every ten minutes within the 1½ hour period of ultrasonication. These steps were to ensure maximum extraction of artemisinin from the *A. annua* cells. The collected hexane extract was evaporated to dryness by using nitrogen gas. Then, 1 ml of acetonitrile was added to the residue. The acetonitrile solution containing artemisinin was filtered with 0.45 µm Millipore before transferring into amber vials (ANSI). From this solution, 20 µl was injected for quantification of artemisinin. Artemisinin was analysed using the method described by ElSohly et al. (1987), but with these modifications, the mobile phase was acetonitrile acetate buffer (70: 30) at a flow rate of 1 ml/min.

Conclusion

In conclusion, continuous subculturing of the callus and cells on the callus induction medium in solid and liquid form could increase the callus and cells biomass at every four weeks and 16 days subculture cycle, respectively. As for the production of artemisinin, cell cultures of *A. annua* were to be harvested at day 12th, whereas for the production of biomass, harvesting of cells should be done before the day 18th. Increasing initial inoculum sizes reduces the time at which the cells achieved maximum growth and influenced the production of artemisinin. The area of *A. annua* cultivation is limited to temperate areas or regions at high altitude. However, with the *in vitro* cell culture technique, artemisinin can be produced from *A. annua* without planting in Malaysia a tropical country which is not suitable for its growth. .

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