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Factors Affecting the Selection of Callus Cell Lines and the Preparation of the Cell Suspension Culture of *Artemisia annua* L.

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Abstract

Artemisinin, an important antimalarial drug against multidrug resistant strains of Plasmodium, can be produced in *Artemisia annua* L. Field production of artemisinin is affected by environmental condition and geographical location. *In vitro* production via cell suspension culture is an alternative mode and cell line selection is important to ensure sustainable production of biomass and artemisinin. Callus cell lines were derived from the leaf explants of five *A. annua* clones grown in two different locations in Vietnam. Thirty-four callus cell lines with consistent growth index (GI) were selected from these five clones and were categorized into fast (GI > 20), intermediate (GI 15 - 20) and slow (GI < 15) growing groups. The selected lines were found to have different morphology in term of colour and texture. The callus texture did affect the cell growth of *A. annua* in which the friable callus type showed faster, consistent and sustainable cell biomass production.

Introduction

Artemisia annua L. is an annual herbaceous plant which has been used for the treatment of intermittent fever in Chinese medicine. It was found to be an important source of artemisinin (Klayman 1985). Artemisinin, an endoperoxide sesquiterpene lactone, was used as artemisinin-based combination therapies (ACTs) due to its rapid parasite clearance and ability to lower the rate of resistance emerges and spreads of malaria parasites (Mutabingwa 2005).

Production of artemisinin from field grown plants was affected by environmental condition, geographical area and time of harvesting (Gupta et al. 2002 and Woerdenbag et al. 1994). Therefore, obtaining artemisinin from field

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grown plant is not consistent. Cell suspension culture technique provides an alternative tool to enhance the production of artemisinin by feeding the precursors and elicitation (Baldi and Dixit 2008). The production of any secondary metabolite from plant cells also depends on the selected cell lines. Although cell suspension culture provides environmental, ecological and climate independent condition for production of bioactive compounds, inconsistent growth rate and productivity is one of the major factors for concern (Kim et al. 2004 and Qu et al. 2006). Plant cell suspension culture often causes genetically instability in terms 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.

This paper is to study the nature of the callus culture of *A. annua* obtained from two geographical locations in Vietnam and to select the consistent productive cell lines for the preparation of cell suspension culture for the production of artemisinin.

Materials and Methods

The seeds of two *Artemisia annua* L. clones (TC1 and TC2) obtained from Institute of Tropical Biology (ITB), Ho Chi Minh City and three clones (dTC1, dTC2 and Highland) from Da Lat, Vietnam were germinated *in vitro* in Plant Tissue and Cell Culture Laboratory, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia. Callus were induced from the leaf explants of the eight weeks old *in vitro* plantlets on MS supplemented with 0.5 g/l casein hydrolysate, 0.5 mg/l NAA and 0.5 mg/l BA. The medium was solidified with 8.0 g/l agar (Chile Agars) and the pH was adjusted to 5.7 prior to autoclaving.

The callii produced from each *A. annua* clones were mixed together as a pool of callus, and 0.5 g of the callus was transferred on to the fresh medium in which each bottle of callus represented one different line (S0). The callus cultures were placed under continuous light with light intensity 32.5 μmol m^2/s at 25 ± 2ºC and the biomass (fresh weight) of callus produced was recorded after four weeks of culture and this was designated subculture one (S1). From this S1 culture, 0.5 g of the callus was again subcultured on to a fresh medium and the callus biomass was again determined after four weeks of culture. This represented subculture S2. The same process was repeated at each subculture cycle and three replicates were used for each cycle. The subculture process was carried out every four weeks until S13.

The biomass produced was recorded and growth index (GI) (Godoy-Hernández and Vázquez-Flota 2005) was calculated at each subculture cycle.

$$\text{Growth Index (GI)} = \frac{\text{Final weight (g)} - \text{Initial weight (g)}}{\text{Initial weight (g)}}$$

The callus lines produced were then categorized into three different groups (fast, intermediate and slow growing) based on the growth index.

The stable lines were then selected for the establishment of cell suspension culture of *A. Annua.* Five callus cell lines from the fast growing group were used for the preparation of cell suspension culture. The cell culture was prepared by transferring 0.5 g of the callus into 100 ml Erlenmeyer flask containing 25 ml fresh liquid medium of the same constituent as the callus proliferation medium. The cells were harvested every 16 days for ten subculture cycles. The GI of cell cultures was recorded at each subculture cycle.

Results and Discussion

Two clones of *A. annua* originating from Ho Chi Minh City and three clones from Da Lat were used in this study. For *A. annua* clones from Ho Chi Minh City, 12 callus cell lines were derived from TC1 clone and seven from TC2 clone. As for *A. annua* clones from Da Lat, 12 callus cell lines were derived from dTC1 clone, 10 from dTC2 clone and 16 from the Highland clone. From a total of 57 callus cell lines produced, only 34 showed consistent GI and were selected. Of these, nine, six, four, eight and seven callus cell lines were derived from TC1, TC2, Highland, dTC1 and dTC2 clones, respectively. These selected callus cell lines were categorized into three different groups, fast, intermediate and slow growing lines, based on their growth capability. The fast growing callus cell lines were those with consistent GI of more than 20 at every subculture cycle from 3rd to 13th subculture cycles. As for intermediate group, the GI range was between 15 and 20. The slow growing group only produced biomass with GI less than 15 (Table 1). Overlapping of the GI curve could be observed between some of the fast and intermediate lines and between the intermediate and the slow growing lines at certain subculture cycles as in Fig. 1 which displayed only some of the selected lines.

The 34 selected callus cell lines were observed to have different morphology (Fig. 2). The callus cell lines derived from Ho Chi Minh areas were yellow, yellowish green or green in color and most the callus lines produced (11 lines) were of the compact type as opposed to friable nature (4 line). The callus lines derived from Da Lat were of many colours (brown, yellow, green and dark green). There were more cell lines of the friable callus type (11 lines) as compared to compact line (8 lines). The results obtained also indicated that the morphology of the callus was not depended on their growth rate but more on the location from where they were derived (Table 1). The clones of *A. annua* used in this

study were obtained from two different geographical locations in Vietnam, Ho Chi Minh City and Da Lat. The conditions of these two locations are distinctly different in term of the sea level, weather and temperature. The growth rate and the artemisinin content of the *ex vitro A. anua* plants planted in Da Lat were significantly higher as compared to Ho Chi Minh City (Thu et al. 2011). The different environmental conditions might affect the growth and contribute to their different callus morphology.

Table 1. Morphology and classification of selected callus cell lines of *A. annua*.

Location	Clone	Cell line category		
		Fast (GI > 20)	Intermediate (GI 15 - 20)	Slow (GI < 15)
Ho Chi Minh	TC1	- green, compact, soft **L1TC1** (22.3 ± 0.6) **L6TC1** (23.8 ± 0.5) - yellowish green, friable, soft **L11TC1** (24.2 ± 0.4) **L12TC1** (26.8 ± 0.6) **L15TC1** (29.1 ± 0.2)	- green, compact, soft **L2TC1** (15.6 ± 0.6) **L3TC1** (19.2 ± 0.5) - yellowish green, friable, soft **L13TC1** (19.1 ± 0.6)	- green, compact, soft **L7TC1** (13.2 ± 0.4)
	TC2	- yellow, semi-compact, soft **L1TC2** (26.7 ± 0.8) **L2TC2** (37.1 ± 0.9) **L4TC2** (26.0 ± 1.2) **L5TC2** (33.9 ± 0.3)	- green, compact, hard **L8TC2** (18.9 ± 0.7) - green, compact, soft **L9TC2** (19.3 ± 0.7)	
Da Lat	Highland	- green, friable, soft **L2Hi** (21.0 ± 0.8) - brown, friable, soft **L19Hi** (21.2 ± 0.8)	- brown, friable, soft **L13Hi** (15.3 ± 0.7)	- brown, friable, soft **L12Hi** (14.2 ± 1.0)
	dTC1	- green, compact, hard **L8dTC1** (33.9 ± 1.3) - brown, spongy, soft **L10dTC1** (28.3 ± 1.5)	- brown, spongy, soft **L3dTC1** (15.2 ± 1.6) **L4dTC1** (17.4 ± 0.6) **L9dTC1** (18.5 ± 1.5) - dark green, compact, hard **L6dTC1**(15.5 ± 0.8)	- brown, spongy, soft **L1dTC1** (10.9 ± 0.8) **L11dTC1** (12.3 ± 0.5)
	dTC2	- yellow, friable, soft **L2dTC2** (28.5 ± 0.9) **L3dTC2** (23.8 ± 0.7) **L5dTC2** (27.3 ± 0.6) **L7dTC2** (26.5 ± 0.7) **L8dTC2** (25.2 ± 0.8) L**10dTC2** (25.5 ± 0.6)	- brown, friable, soft **L9dTC2** (17.5 ± 0.7)	

The values shown are mean value of GI ± standard error.

In this study, five fast growing cell lines of compact and friable callus types were used for the preparation of cell suspension cultures of *A. annua.* The friable callus lines (L11TC1, L2dTC2, and L2Hi) showed better growth as compared to

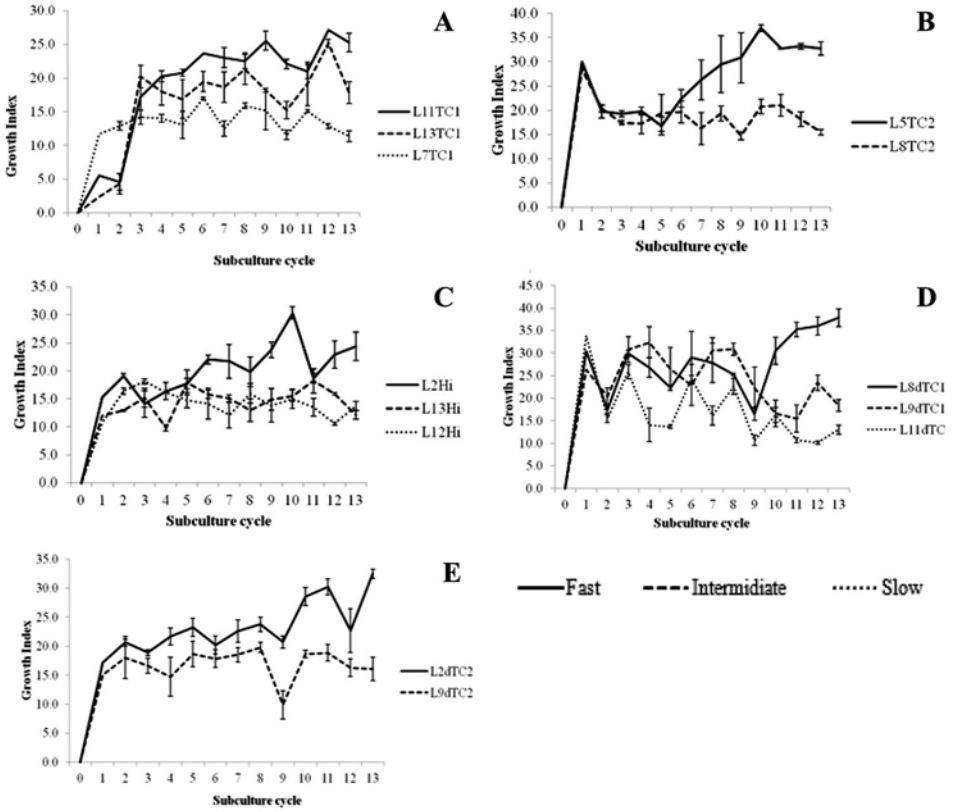

Fig. 1. The growth of callus cell lines from each category (fast, intermediate and slow growing) of five clones (A. TC1, B. TC2, C. Highland, D. dTC1 and E. dTC2).

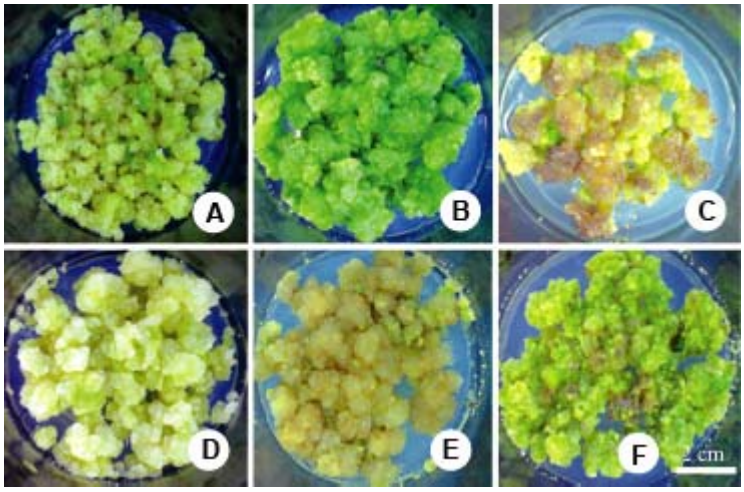

Fig. 2. Type of callus observed in different cell lines: A. Yellowish green, friable and soft. B. Green, compact and hard. C. Brown, spongy and soft. D. Yellow, semi-compact and soft. E. Brown, friable and soft. F. Green, compact and soft.

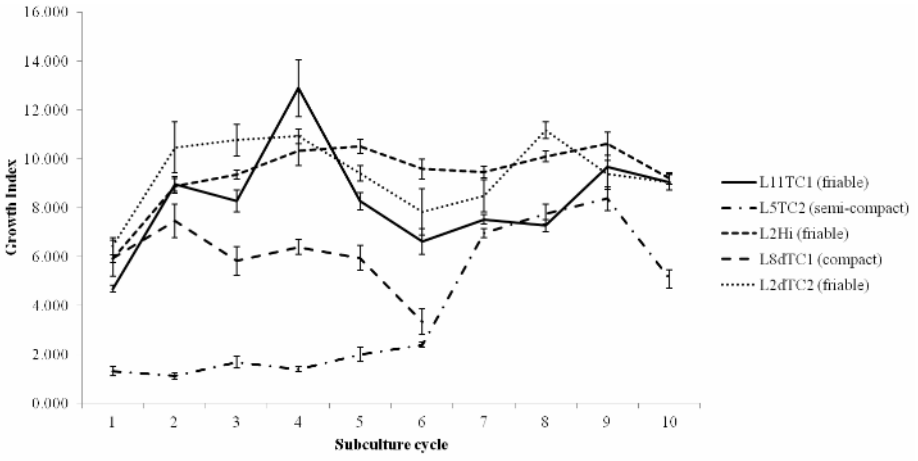

Fig. 3. The growth pattern of five fast growing lines in cell suspension culture.

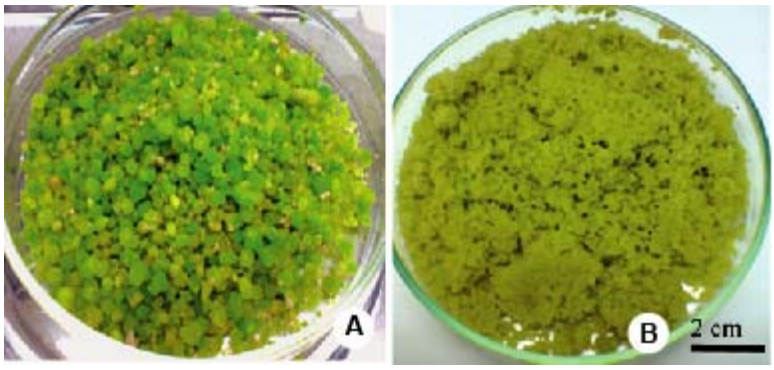

Fig. 4. Cells derived from: A. compact callus cell line (L8dTC1) and B. friable callus cell line (L11TC1).

compact (L8dTC1) and semi-compact (L5TC2) callus lines (Fig. 3). The growth of the compact cell lines was found not to be consistent and the cells provided were made up of big cell aggregates (Fig. 4A). The growth of the friable cell lines remained consistent and the cell cultures consisted of fine cells (Fig. 4B). Moreover, the growth of compact cell line (L8dTC1) was retarded and not sustainable, thereby indicating that the callus morphology did play an important role in the selection of cell lines of *A. annua* for the preparation of a sustainable cell culture.

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