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Effect of Plant Growth Regulators and Subculture Frequency on Callus Culture and the Establishment of *Melastoma malabathricum* Cell Suspension Cultures for the Production of Pigments

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Abstract: Friable callus of *Melastoma malabathricum* could be induced from the leaf explants on MS medium supplemented with 1 mg L⁻¹ N6-benzylaminopurine (BA) + 6 mg L⁻¹ 1-naphthaleneacetic acid (NAA). Continuous subculturing of the friable calluses on the callus induction medium could increase the callus biomass at every 4 weeks subculture cycle. The production of callus biomass became stable with a growth index of 10 or more after the tenth subculture cycles. The cell suspension culture of *M. malabathricum* was initially established by culturing the leaf-derived friable callus in the liquid callus induction medium. The cells subsequently grew healthily and maintained well in MS liquid medium supplemented with 0.25 mg L⁻¹ BA and 0.5 mg L⁻¹ NAA. The growth kinetics of *M. malabathricum* cells followed a general growth pattern of a sigmoid curve. However, browning occurred when the cultures reached the highest fresh cell mass. In order to maintain healthy cultures, subculturing had to be done before browning occurred. After nine days of culture, cell inoculums of 0.75 and 1.0 g could produce fresh cell mass of 5.560 and 5.147 g, respectively. On the 15th day, cell cultures with initial inoculums of 0.25 and 0.5 g produced fresh cell mass of 5.416 and 6.150 g, respectively with cell mass increased of 22 and 15 fold, respectively. To maintain the cultures, an initial inoculum of 0.25 to 0.5 g of cells could be used and subcultured between 9 and 15 days after initial inoculation.

Key words: Anthocyanin, friable callus, inoculum, 1-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), N6-benzylaminopurine (BA), pigmented callus

INTRODUCTION

Plants are the main natural resources for many pigments used by mankind since the very early days, either in dyeing of textiles or as food colouring. *Melastoma malabathricum*, belongs to the Melastomaceae family, has been found to be an important dye-producing plant. The roots are used in mixtures for dyeing red, the leaves for purple and the fruits used for dyeing cloth black (Lemmens and Wulijarni-Soetjito, 1991). The colours in the plant are due to the presence of anthocyanins. Though the anthocyanins are easily recognized as flower pigments, their occurrence is not restricted to flowers but include all parts of the plant.

M. malabathricum grows very well in the tropical and sub-tropical countries especially in South-East Asian countries, Central Asia, Southern part of China, Taiwan, Australia and the remote islands of the Pacific. It is

commonly known as Singapore Rhododendron or Senduduk in Singapore and Malaysia, Mang kre or Mang re in Thailand and Kluruk or Senggani in Java (Corner, 1997). It is commonly found in waste lands where the soils are poor and acidic.

Besides its importance as a source of pigments, parts of the *M. malabathricum* plants have been widely used in traditional medicine. Every parts of the plant including the roots can be used as the material source for the preparation of traditional medicine and as natural food colourant due to the presence of anthocyanins (Janna *et al.*, 2006). Jaganath and Ng (2000) reported that puerperal disease and infectious diarrhea could be treated by eating the raw senduduk leaves. Lately, anthocyanins had been found to have an antioxidant activity which was equivalent to or even higher than a common vitamin E analog. It was also proven to have anti-inflammation and anti-nociceptive effect in mice (Sulaiman *et al.*, 2004).

These activities may account for some of the beneficial effects derive from the consumption of fruits and vegetables that are high in anthocyanins against coronary and heart diseases (Zhang *et al.*, 2005). With the increasing public awareness on natural health food, we focus on a study to produce natural dye and food supplement from *M. malabathricum* via *in vitro* cell culture technology. Aobchey *et al.* (2002) had successfully produced natural red dye (antraquinone) from the root cell cultures of *Morinda angustifolia* Roxb. var. *scabridula* Craib. In this study, we report on the selection of the elite cell lines and evaluating the cultural condition that affect the establishment of the cell suspension culture of *M. malabathricum* for the production of anthocyanin pigments.

MATERIALS AND METHODS

Establishment of *in vitro* plantlets: *In vitro* plantlets were obtained by *in vitro* germinations of the seeds. The germination of the seeds was carried out in June 2006. Fruits were collected from the open field around Penang Island, Malaysia. The outer layers of the fruit pulps were removed and the remainder part was surface sterilized with 20% (v/v) Clorox®, a commercial bleach, with the addition of three drops of Tween-20 (polyoxyethylene sorbitan monolaurate) for 20 min with continuous agitation on a rotary shaker at 120 rpm. After three rinses with sterile distilled water, the ovaries were cut into 6-8 pieces longitudinally. The ovary walls were removed and lumps of seeds were placed on solidified basic MS medium (Murashige and Skoog, 1962) in 300 mL culture vessels. The pH of the medium was adjusted to 5.7 before autoclaving for 11 min at 121°C under a pressure of 1.05 kg cm⁻² (Tommy Autoclave SS-325). The cultures were placed in a culture room maintained at 23 ±2°C under continuous illumination with cool white florescent tubes at a light intensity of 32.5 µE m⁻² sec⁻¹ until the seeds germinated to produce seedlings. Leaves of eight weeks old *in vitro* seedlings were used as explants for the induction of callus.

Effect of plant growth regulators on induction of callus

Effect of 2,4-D and NAA: The transversely cut leave pieces (4-5 mm) were inoculated into 150 mL culture bottles containing MS medium supplemented with (0-10 mg L⁻¹) 2,4-dichlorophenoxyacetic acid (2,4-D) and 1-naphthaleneacetic acid (NAA) (0, 0.5, 1.0 and 2.0 mg L⁻¹) using 6×4 factorial experiment in a randomized

complete block design. Three leaf explants were inoculated into each culture vessel and 15 experimental units were used for each combination treatment. Fresh biomass of callus formed was determined after seven weeks of culture. The data were analyzed using two-way ANOVA followed by Duncan's multiple range test (DMRT) at p = 0.05.

Effect of BA and NAA: The leaves of the 8 weeks old *in vitro* plantlets were cut transversely into pieces with width of 4-5 mm. The leaf explants were cultured on solidified MS medium supplemented with different concentrations (0-10 mg L⁻¹) of N6-benzylaminopurine (BA) and NAA. The selection of the best combination of plant growth regulators (BA and NAA) for friable callus induction was carried out in three stages.

In the first stage, the leaf explants were cultured on MS medium supplemented with a combinations of 2-6 mg L⁻¹ BA and 4-8 mg L⁻¹ NAA in 3×3 factorial experiment in a complete randomized design. In the second stage, the concentration of plant growth regulators supplemented into the MS medium were adjusted to 0-4 mg L⁻¹ BA and 2-6 mg L⁻¹ NAA using also 3×3 factorial experiment in a complete randomized design. In the third stage, the leaf explants were inoculated on MS medium supplemented with 0 and 1 mg L⁻¹ BA and 5-8 mg L⁻¹ NAA in 2×4 factorial experiment in a complete randomized design. Three leaf explants were used for each experimental unit and 15 units for each combination treatment. Fresh biomass of callus formed at each stage and its physical morphology were observed and recorded after seven weeks of culture. The data for each stage of experiment were analyzed using one-way ANOVA followed by Duncan's Multiple Range Test (DMRT) for mean comparison at p = 0.05.

Effect of subculture on callus growth: The leaf explants (0.5×1 cm) were cultured on MS medium supplemented with 1 mg L⁻¹ BA and 6 mg L⁻¹ NAA, the best callus induction medium, for seven weeks. Since the induced callus started to turn brown before the end of 7th week, the calluses were hence subcultured at every four weeks interval. As sufficient amount of friable callus for a minimum of 6 replicates could only be collected after 3 subcultures the effect of subculture could only be determined from the 4th subculture cycle. With an initial mass of 1.0 g of callus, the amount of callus produced and its average growth index was determined after each subculture cycle until the 26th subculture cycles. The cultures were incubated in the culture room

maintained at $23 \pm 2^\circ\text{C}$ with continuous light intensities of $32.5 \mu\text{E m}^{-2} \text{sec}^{-1}$. The fresh callus biomass and the growth index were subsequently determined randomly during subculturing process. Eight replicates were used for each subculture cycle. Fresh callus biomass was determined at the end of 4 weeks culture using the digital weighing scale (Denver Instrument XL-410, USA). The Growth Index (GI) was determined as:-

$$\text{GI} = \frac{\text{Final biomass} - \text{Initial biomass}}{\text{Initial biomass}}$$

Establishment of cell suspension culture: Half a gram of friable callus obtained from leaf explants was first transferred into 100 mL Erlenmeyer conical flasks containing 20 mL liquid MS medium supplemented with 1 mg L^{-1} BA + 6 mg L^{-1} NAA. Six replicates were used for the establishment of cell suspension cultures. The cultures were placed on an orbital shaker at 120 rotations per minute (120 rpm) under continuous illumination with light intensities of $20 \mu\text{E m}^{-2} \text{sec}^{-1}$ at $23 \pm 2^\circ\text{C}$. At the end of two weeks, the cells were harvested from the liquid medium by filtering it using an air suction pump. The fresh cell mass obtained was weighed using the digital weighing scale (Denver Instrument XL-410, USA).

As the liquid MS medium supplemented with 1 mg L^{-1} BA + 6 mg L^{-1} NAA was found to be not suitable for cell suspension culture, an experiment was carried out to determine the suitable cell proliferation medium using factorial design. Half a gram of friable callus was transferred into 100 mL conical flasks containing 20 mL liquid medium with three reducing stages of concentrations of plant regulators. In the first stage, 1 and 2 mg L^{-1} BA and 2 and 4 mg L^{-1} NAA were supplemented into the MS medium. In the second stage, 0.5 and 1 mg L^{-1} BA and 1 and 2 mg L^{-1} NAA were tested. Lastly, 0.125 and 0.25 mg L^{-1} BA and 0.25 and 0.5 mg L^{-1} NAA were evaluated for their suitability. Each combination treatment was carried out with seven replicates.

The cells were harvested after 14 days of culture and the fresh cell mass was determined. The cell biomass data at each stage were analyzed using a one-way ANOVA followed by Duncan's Multiple Range Test (DMRT) to determine the best plant growth regulator combination at $p = 0.05$. Morphology of the cell aggregates was also observed and recorded.

To obtain uniform cell suspension cultures of *M. malabathricum* L., the cells cultured in the best proliferation medium (MS + 0.25 mg L^{-1} BA + 0.5 mg L^{-1}

NAA) were filtered using sterilized stainless steel sieve with $850 \mu\text{m}$ pore to separate small cell aggregates from the bigger ones. Individual cells or small cell aggregates that passed through the sieve (with diameter less than $850 \mu\text{m}$) were subsequently filtered using an air suction pump to remove the medium and subcultured into fresh proliferation medium. Subculture was done every 14 days. All cultures were placed on an orbital shaker at 120 rpm in a culture room maintained at $23 \pm 2^\circ\text{C}$ under continuous illumination with cool white florescent tubes at a light intensity of $20 \mu\text{E m}^{-2} \text{sec}^{-1}$.

Determination of the growth kinetics of cells: The growth kinetics of cells was determined over a period of 24 days using 4 different initial inoculum sizes: 0.25, 0.5, 0.75 and 1.0 g in 100 mL Erlenmeyer flasks containing 20 mL of MS medium supplemented with 0.25 mg L^{-1} BA and 0.5 mg L^{-1} NAA, the optimum cell culture medium. Throughout the period of 24 days the cell biomass of six replicates was taken every three days. From the growth kinetics, suitable initial inoculum size and the best subculture cycle interval were determined.

RESULTS AND DISCUSSION

More than 90% of aseptic seeds of *Melastoma malabathricum* could be obtained using the suggested surface-sterilization protocol and 94.4% of the seeds germinated after 22 days culturing on the basal MS medium. The seedlings were allowed to grow in the same medium without plant growth regulators for 8 weeks (Fig. 1) to be used as the plant materials for the establishment of callus and cell suspension cultures. The supplement of plant growth regulators especially the cytokinin into the culture medium will later encourage



Fig. 1: Eight weeks old *in vitro* seedlings of *Melastoma malabathricum*

organogenesis rather than callus formation. Hence, the *in vitro* *M. malabathricum* seedlings were cultured on basal MS medium so that the leaf explants to be used as plant materials for callus formation would not undergo organogenesis.

Preliminary study done by Low (2001) showed that more than 75% of the *M. malabathricum* leaf explants produced callus when they were culture on gelled MS medium supplemented with 0-4 mg L⁻¹ 2,4-D with the addition of 2 mg L⁻¹ NAA. Very little or no callus was induced when the amount of NAA added into the basal MS medium was more than 4 mg L⁻¹. Present result indicated that the addition of only NAA as low as 0.5 mg L⁻¹ was sufficient for the induction of callus from the leaf explants of *M. malabathricum* and 0.75 g of callus was produced after 7 weeks of culture. The amount of callus induced gradually reduced as the concentration of NAA added was increased and only 0.2 g of callus was formed from the leaf explants within the same duration when they were cultured on MS supplemented with 2 mg L⁻¹ NAA. MS medium supplemented with low level of 2,4-D (2 mg L⁻¹) without NAA could also stimulate callus growth (0.6 g) from the leaf explants within the same period. The presence of high level of 2,4-D (4 mg L⁻¹ and more) in the culture medium reduced the formation of callus. The presence of both NAA and 2,4-D in the culture medium did not support consistent growth of the callus (Fig. 2). This indicated that either NAA or 2,4-D could be used for the induction of callus from *M. malabathricum* leaf explants. Generally, for most herbaceous plant only one type of auxin is sufficient and required to be incorporated into the nutrient medium for the induction of callus from explants (George and Sherrington, 1984). Luthfi *et al.* (2003) had successfully induced callus formation from the leaf explants of *Eurycoma longifolia*, a woody plant, using MS medium supplemented with only NAA but at high level (10 mg L⁻¹). While Kattipongpatana(1998) reported that callus could be induced from the leaf and stem explants of *Solanum aviculare* cultured on MS medium supplemented with 4.52 µM 2,4-D. Thomas and Maseena (2006) also found that highest callus production could be obtained from the leaf and nodal explants of *Cardiospermum halicacabum* using MS medium with the addition of only 2,4-D (5 µM).

In some cases, a cytokinin was also added together with the auxin to the medium for stimulation of callus growth. For instance, callus was initiated from the shoot tips of *Zingiber officinale* using MS medium supplemented with combination of NAA (0.1 mg L⁻¹) and

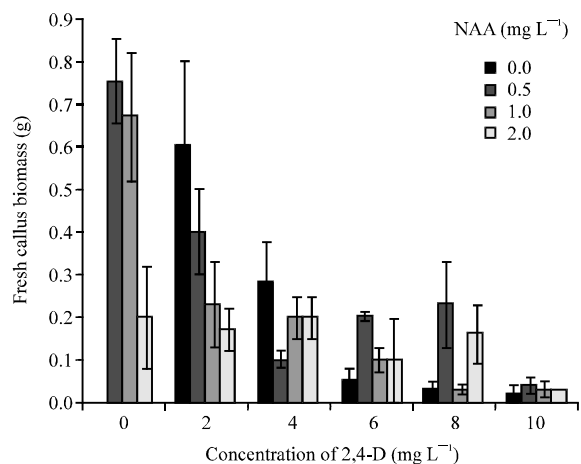


Fig. 2: Effect of 2,4-D (0-10 mg L⁻¹) and NAA (0, 0.5, 1.0, and 2.0 mg L⁻¹) supplemented into basic MS medium on production of callus from the leaf explants of *M. malabathricum* after 7 weeks of culture

kinetin (1-2 mg L⁻¹) (Jamil *et al.*, 2007). High percentage of callus was induced from the root tips of two varieties of garlic on MS medium supplemented with 1.5 mg L⁻¹ 2,4-D and 5 mg L⁻¹ kinetin (Khan *et al.*, 2004). The best medium for callus production of *Silybum marianum* was B5 medium supplemented with 0.05 mg L⁻¹ BA and 0.5 mg L⁻¹ 2,4-D (Cimino *et al.*, 2006). Since both NAA or 2,4-D incorporated into MS medium did not induce satisfactory amount of callus from *M. malabathricum* leaf explants, we decided to supplement the MS medium with combination of both auxin (NAA) and cytokinin (BA). Present results indicated that 75-83% of the leaf explants produced callus at the cutting edges of the leaf explants when they were cultured on MS medium supplemented with 2-6 mg L⁻¹ BA and 4-8 mg L⁻¹ NAA. The callus started to form by the end of 5th week with the most callus formed on MS basic medium supplemented with 2 mg L⁻¹ BA and 4 mg L⁻¹ NAA with a fresh callus biomass of 2.06±1.27 g at the end of 7th week culture. The callus produced on this medium was a mixture of friable and compact in nature. In this study obtaining a soft and friable callus is preferable compared to the compact ones as only the friable callus will be suitable for the establishment of the cell suspension culture. The amount of callus produced was drastically reduced when the concentration of BA and NAA added into the culture medium was increased (Table 1). Hence the amount of BA and NAA used was further modified and adjusted in

Table 1: Effects of different concentrations of BA and NAA supplemented into MS medium on the induction and morphology of callus from the leaf explants of *M. malabathricum* after 7 weeks of culture

Experimental stage	Growth regulators (mg L ⁻¹)		Mean fresh mass (g) ±SD	Morphology of callus
	BA	NAA		
First	2	4	2.06±1.27a	Friable+compact
	2	6	0.25±0.16cd	Friable
	2	8	0.31±0.28cd	Friable
	4	4	0.81±0.45b	Friable
	4	6	0.36±0.20cd	Compact
	4	8	0.12±0.10d	Compact
	6	4	0.58±0.66bc	Friable
	6	6	0.14±0.11d	.*
	6	8	0.13±0.11d	.*
	0	2	0.82±0.22a	Friable [#]
Second	0	4	0.36±0.26cd	Friable [#]
	0	6	0.19±0.07de	Friable [#]
	2	2	0.43±0.19c	Compact
	2	4	0.53±0.30bc	Compact
	2	6	0.21±0.09de	Compact
	4	2	0.67±0.41ab	Compact
	4	4	0.22±0.19de	Compact
	4	6	0.16±0.07e	Compact
	0	5	0.86±0.16b	Friable
	0	6	0.46±0.15c	Roots formed
Third	0	7	0.35±0.12d	Roots formed
	0	8	0.15±0.12d	Compact
	1	5	0.63±0.37bc	Friable
	1	6	1.58±0.72a	Friable
	1	7	0.36±0.17cd	Friable
	1	8	0.54±0.28c	Friable

Mean values followed by the same alphabet within the same experimental stage are not significantly different (DMRT, $p = 0.05$); Morphology of callus is considered friable or compact if more than 50% of it is friable or compact respectively, .*Callus was too little for identification, [#]Callus showed red pigmentation at the end of 7th week

the second stage of callus induction study. Friable callus with the highest mean fresh weight of 0.82 ± 0.22 g was obtained from MS basic medium supplemented with 2 mg L^{-1} NAA after seven weeks of culture. At this stage we could obtain the suitable friable callus ideal for the preparation of the cell suspension culture of *M. Malabathricum* but the amount produced was still not satisfactory. The amount of supplemented plant growth regulators (BA and NAA) was further adjusted in the third stage. MS basic medium supplemented with 1 mg L^{-1} BA and 6 mg L^{-1} NAA recorded a better callus yield with fresh biomass of 1.58 ± 0.72 g after 7 weeks of culture (Table 1).

The callus biomass was found to increase at each subculture interval starting from the 4th subculture cycle. The increased amount of callus produced was 2.5 times that of initial inoculum at the 4th subculture cycle and continued to increase until the growth index was 11.4 after 10 subculture cycles. After the 10th subculture cycles, random checking on the growth index of the *M. malabathricum* callus showed that an average growth

Table 2: Effect of subculture on biomass and growth of *M. malabathricum* callus on solid MS medium supplemented with 1 mg L^{-1} BA and 6 mg L^{-1} NAA

No. of subculture cycles	Callus fresh biomass at the end of subculture cycle (g) ±SD	Average growth index (GI)
4	3.6±0.8	2.5
5	4.1±1.2	2.9
6	5.3±2.5	4.2
8	8.2±3.2	7.1
10	14.494	11.4
Subsequent	11.537	10.4
subcultures	13.772	12.4
(check randomly)	12.990	11.3

Fig. 3: Loose pigmented callus culture of *M. malabathricum*

index of 10.4 to 12.4 could be obtained (Table 2). The callus culture was well maintained with aggregates of loose red pigmented cells often seen occurred at the top or at the edges of the culture (Fig. 3). Present result was similar to those findings obtained by Banthorpe *et al.* (1995). They reported the accumulation of pigments appeared in slow-growing or senescent cultures of *Lavandula angustifolia*. When pigmented calluses were cultured onto fresh callus proliferation medium, MS medium supplemented with 1 mg L^{-1} BA and 6 mg L^{-1} NAA, the cells started to actively divide and pigmentation disappeared. Pigmentation could recur towards the end of each subculture cycle of four weeks interval. An establishment of pigmented callus line of *M. malabathricum* L. could not be maintained consistently. Fast growing callus lines were then selected on non-pigmented callus. However, Meyer and Van Staden (1995) reported that the red callus of *Oxalis linearis* did not revert back to non-pigmented stage.

When the friable leaf callus was transferred from solid medium into liquid medium of the same concentration of plant regulators (MS + 1 mg L^{-1} BA + 6 mg L^{-1} NAA), an initial inoculum of 0.5 g callus resulted

in the production of only 0.27 ± 0.06 g cell biomass at the end of 14 days of culture. The reduction in cell biomass indicated that the cells were dead. After the medium was removed using the air suction pump, the cells that remained on the filter paper were sticky. This suggested that the medium composition was not suitable for the preparation of cell suspension culture of *M. malabathricum* L. Hence, the amount of plant growth regulators in the culture medium was reduced step by step.

In the first stage of adjusting the plant growth regulators content in the culture medium, all combinations of plant growth regulators (1.0 and 2.0 mg L⁻¹ NAA and 2.0 and 4.0 mg L⁻¹ BA) supplemented into the MS medium resulted in very little cell yield after 14 days of culture. Cell aggregates collected were black and sticky which indicated the occurrence of cell death. All combinations of plant regulators were found to be not suitable for the preparation of the cell suspension culture of *M. malabathricum*. In the second stage of plant growth regulators adjustment, MS medium supplemented with 0.5 mg L⁻¹ BA and 1 or 2 mg L⁻¹ NAA induced better cell biomass yield but was also not satisfactory. The cells grew in the MS medium supplemented with 1.0 mg L⁻¹ BA and 1.0 or 2.0 mg L⁻¹ NAA were also black and sticky which was a clear indication of cell death. In the third stage, the fresh cell biomass obtained at the end of 14 days of cell cultures maintained in MS medium with the addition of 0.125 – 0.5 mg L⁻¹ BA and 0.25 – 0.5 mg L⁻¹ NAA was better compared to the earlier two stages. Cell aggregates were not sticky and the occurrence of cell death was much reduced. Fresh cell biomass obtained at the end of 14 days was not significantly different. The key basis for best medium selection laid down to the morphology of cell aggregates. Though MS medium supplemented with 0.5 mg L⁻¹ BA and 0.5 mg L⁻¹ NAA supported the highest cell biomass, the cell aggregates tend to stay in bigger clumps. MS basal medium supplemented with 0.25 mg L⁻¹ BA and 0.5 mg L⁻¹ NAA though produced slightly less cell biomass but produced smaller cell aggregates (Table 3). In cell suspension culture, obtaining smaller cell aggregates was more desirable. Thus MS basal medium supplemented with 0.25 mg L⁻¹ BA and 0.5 mg L⁻¹ NAA was chosen as the best medium for the preparation of cell suspension culture of *M. malabathricum* and all the cell cultures were subsequently maintained in this medium composition, MS + 0.25 mg L⁻¹ BA + 0.5 mg L⁻¹ NAA.

The growth kinetics of the *M. malabathricum* cells cultured in liquid MS + 0.25 mg L⁻¹ BA + 0.5 mg L⁻¹ NAA followed a general pattern of sigmoid curves based on the fresh cell mass (Fig. 4). From the four initial inocula tested,

Table 3: Average fresh cell biomass of *M. malabathricum* after two weeks of culture in MS medium supplemented with different concentration (mg L⁻¹) of BA and NAA

Stages	Plant growth regulators (mg L ⁻¹)		Average fresh cell mass at the end of culture period (g±SD)
	BA	NAA	
First	1.000	2.00	0.079±0.020
	1.000	4.00	0.050±0.020
	2.000	2.00	0.075±0.013
	2.000	4.00	0.074±0.017
Second	0.500	1.00	0.133±0.039
	0.500	2.00	0.149±0.074
	1.000	1.00	0.113±0.055
	1.000	2.00	0.126±0.036
Third	0.125	0.25	0.306±0.180b
	0.125	0.50	0.284±0.134b
	0.250	0.25	0.456±0.198a
	0.250	0.50	0.329±0.125b

Means followed by the same alphabet on the third stage of experiment were not significantly different (Duncan's multiple range test, $p = 0.05$)

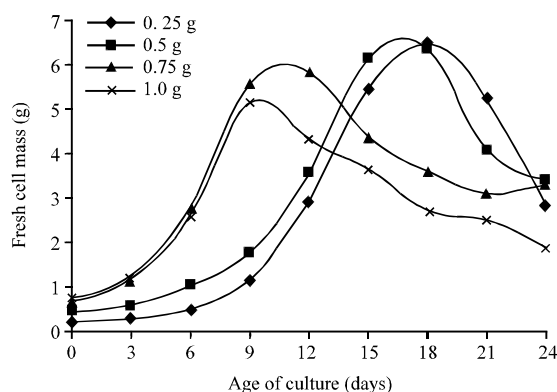


Fig. 4: The growth kinetics of *M. malabathricum* using different cell inoculum size in MS + 0.25 mg L⁻¹ BA + 0.5 mg L⁻¹ NAA

generally two growth patterns were observed. Cell cultures with initial inocula of 0.25 and 0.5 g went through a period of lag phase before active cell division took place. Cell cultures with initial inocula of 0.75 and 1.0 g, on the other hand, went through very short lag phase. Active cell growth took place immediately after the cells were transferred into fresh liquid MS medium supplemented with 0.25 mg L⁻¹ BA and 0.5 mg L⁻¹ NAA.

After a lag phase of 6 days, cell cultures with an initial inoculum of 0.25 g started to grow and divide actively, entering its exponential growth phase on day 9 and reached its maximum fresh cell mass of 6.50 g, with a growth index of 25.0 on day 18. Its growth rate immediately became negative after day 18. Cell cultures with initial inoculum of 0.5 g started to grow and divide actively after a lag phase of 3 days. The cultures showed exponential growth from day 9 to day 15. Its growth rate decreased after day 15 before achieving its maximum fresh cell mass of 6.35 ± 0.88 g and a growth index of 11.7 on day 18. After day 18, its growth rate became negative.



Fig. 5: Purplish-red pigmented cells of *M. malabathricum* cultured in MS + 0.25 mg L⁻¹ BA + 0.5 mg L⁻¹ NAA incubated at 23±2°C



Fig. 6: Dried pigmented cells of *M. malabathricum*

For cell cultures with initial inocula of 0.75 and 1.0 g, growth rates were low for the first three days. Both cultures were in their exponential phases from day 3 to day 9, a duration of 6 days, as compared to a duration of exponential phase of 9 days exhibited by the other two sets of cultures. The growth rate of cultures with an initial inoculum of 0.75 g decreased slightly before reaching its highest fresh cell mass of 5.84 g and a growth index of 6.8 on day 12. For cultures with an initial inoculum of 1.0 g the growth rate became negative immediately after achieving the highest fresh cell biomass of 5.15 g and a growth index of 4.1 at day 9. For all cultures, browning was noticed when the cultures entered their stationary phases. Thus to maintain the cell cultures of *M. malabathricum*, an initial inoculum of 0.25 to 0.5 g could be used and subcultured between 9 and 15 days of culture.

When the cell cultures of *M. malabathricum* were incubated at low temperature of 23±2°C, purplish-red pigments were produced in the cell suspension culture (Fig. 5). The pigment remained stable in the air-dried cells

(Fig. 6) and anthocyanins extracts could be obtained by soaking the dried cells overnight in acidic ethanol solvent.

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REFERENCES

- Aobchey, P., S. Sriyam, W. Praharnriporab, S. Lhieochaiphant and S. Phutrakul, 2002. Production of red pigment from the root of *Morinda angustifolia* Roxb. var. *scabridula* by root cell culture. Chiang Mai Univ. J., 1: 66-78.
- Banthorpe, D.V., M.J. Bates and M.J. Ireland, 1995. Stimulation of accumulation of terpenoids by cell suspensions of *Lavandula angustifolia* following pre-treatment of parent callus. Phytochemistry, 40: 83-87.
- Comer, E.J.H., 1997. Wayside Trees of Malaya: Vol. II. 4th Edn. Malayan Nature Society, Kuala Lumpur.
- Cimino, C., S.V. Cavalli, F. Spina, C. Natalucci and N. Priolo, 2006. Callus culture for biomass production of milk thistle as a potential source of milk clotting peptidases. J. Biotechnol., 9: 237-239.
- George, E.F. and P.D. Sherrington, 1984. Plant Propagation by Tissue Culture Handbook and Dictionary of Commercial Laboratories. Exegetics Limited, Eversley, Basingstoke, Hants, England.
- Jaganath, I.B. and L.T. Ng, 2000. Herbs: The Green Pharmacy of Malaysia. Vinpress Sdn. Bhd. and Mardi.
- Jamil, M., J.K. Kim, Z. Akram, S.U. Ajmal and E.S. Rha, 2007. Regeneration of ginger plant from callus culture through organogenesis and effect of CO₂ enrichment on the differentiation of regenerated plant. Biotechnology, 6: 101-104.
- Janna, O.A., A. Khairul, M. Maziah and Y. Mohd, 2006. Flower pigment analysis of *Melastoma malabathricum*. Afr. J. Biotechnol., 5: 170-174.
- Kattipongpatana, N., R.S. Hock and J.R. Porter, 1998. Production of solasodine by hairy root, callus and cell suspension cultures of *Solanum aviculare* Forst. Plant Cell Tissue Org. Cult., 52: 133-143.
- Khan, N., M.S. Alam and U.K. Nath, 2004. *In vitro* regeneration of garlic through garlic culture. J. Biol. Sci., 4: 189-191.
- Lemmens, R.H.M.J. and N. Wulijarni-Soetjipto, 1991. Plant Resources of South-East Asia PROSEA No. 3: Dye and tannin-producing plants. PROSEA Foundations, Bogor, Indonesia.

- Low, P.H., 2001. Penghasilan anak benih *in vitro* dan penyediaan ampaian sel *Melastoma malabathricum* L. B.Sc. Thesis, Universiti Sains Malaysia, Penang, Malaysia.
- Luthfi, A.M.S., L.K. Chan and P.L. Boey, 2003. Selection of cell source and the effect of pH and MS macronutrients on biomass production in cell cultures of Tongkat Ali (*Eurycoma longifolia* Jack). J. Plant Biotechnol., 5: 131-135.
- Meyer, H.J. and J. Van Staden, 1995. The *in vitro* production of an anthocyanin from callus culture of *Oxalis linearis*. Plant Cell Tissue Org. Cult., 40: 55-58.
- Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiol., 15: 473-497.
- Sulaiman, M.R., M.N. Somchit, D.A. Israf, Z. Ahmad and S. Moin, 2004. Antinociceptive effect of *Melastoma malabathricum* ethanolic extract in mice. Fitoterapia, 75: 667-672.
- Thomas, T.D. and E.A. Maseena, 2006. Callus induction and plant regeneration in *Cardiospermum halicacabum* Linn. An important medicinal plant. Sci. Hortic., 108: 332-336.
- Zhang, Y., S.K. Vareed and M.G. Hair, 2005. Human tumor cell growth inhibition by nontoxic anthocyanidins, the pigments in fruits and vegetables. Life Sci., 76: 1465-1472.