

# Effects of abiotic stress on biomass and anthocyanin production in cell cultures of *Melastoma malabathricum*

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## ABSTRACT

Plant cell cultures could be used as an important tool for biochemical production, ranging from natural coloring (pigments) to pharmaceutical products. Anthocyanins are becoming a very important alternative to synthetic dyes because of increased public concern over the safety of artificial food coloring agents. Several factors are responsible for the production of anthocyanin in cell cultures. In the present study, we investigate the effects of different environmental factors, such as light intensity, irradiance (continuous irradiance or continuous darkness), temperature and medium pH on cell biomass yield and anthocyanin production in cultures of *Melastoma malabathricum*. Moderate light intensity (301 - 600 lux) induced higher accumulation of anthocyanins in the cells. The cultures exposed to 10-d continuous darkness showed the lowest pigment content, while the cultures exposed to 10-d continuous irradiance showed the highest pigment content. The cell cultures incubated at a lower temperature range ( $20 \pm 2$  °C) grew better and had higher pigment content than those grown at  $26 \pm 2$  °C and  $29 \pm 2$  °C. Different medium pH did not affect the yield of cell biomass but anthocyanin accumulation was highest at pH 5.25 - 6.25.

**Key terms:** irradiance, medium pH, photoperiod, temperature, *Melastoma malabathricum*

## INTRODUCTION

*Melastoma malabathricum* L. (Family Melastomaceae), is a common shrub that grows in previously cleared land and waste places. This plant is an important dye-producing plant. Besides having bright attractive colours and high water solubility, anthocyanins were found to possess antioxidant, anti-inflammatory, anticarcinogenic, antiatherogenic, antibacterial and antiviral properties (Mazza 2000). The demand for natural food colorants in place of synthetic colorants is increasing worldwide. Anthocyanin is one of the important natural dyes, with high market value (US\$ 1250- 2000/ kg) (Delgado-Vargas and Parades-Lopez 2003). The potential to produce anthocyanins through plant cell cultures is being considered as a valuable alternative to the use of synthetic dyes. Plant cell cultures

can provide a year-round supply of the product under controlled conditions (Deroles et al., 2006). Production of anthocyanins via plant cell cultures from various plant species has been successful, such as with *Catharanthus roseus* (Filippini et al., 2003), strawberry (Mori et al., 1993) and purple sweet potato (Terahara et al., 2004). Hence, the potential to produce anthocyanins from *M. malabathricum* through plant cell cultures is a valuable alternative.

Our previous study indicated that anthocyanin could be produced from the callus and cell suspension cultures of *M. malabathricum* (Chan et al., 2008). To optimize the production of anthocyanin we realized that abiotic factors play an important role on cell biomass yield and anthocyanin production in cell suspension cultures of *M. malabathricum*. Therefore, the present study aims to investigate the

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effect of environmental factors i.e., light, photoperiod, temperature and medium pH on cell biomass yield and anthocyanin production of *M. malabathricum*.

#### MATERIAL AND METHODS

##### *Establishment of stable cell suspension cultures of M. malabathricum*

Initiation and establishment of cell suspension culture was based on the method described by Chan et al (2008). The cells were subcultured at 9-day intervals and maintained in liquid Murashige & Skoog (1962) MS medium, supplemented with 0.25 mg dm<sup>-3</sup> benzyladenine (BA) and 0.5 mg/L NAA, the cell proliferation medium according to a previous study by Chan et al (2008). A sterile stainless steel sieve, with 850  $\mu$ m pore size, was used to separate the cells during subculture. Individual cells or small cell aggregates that passed through the sieve (with a diameter < 850  $\mu$ m) were subsequently filtered using an air suction pump (Aspirator 3A-S-Eyela, Raikakikai Co. Ltd, Tokyo) to remove the medium. The cell cultures were placed on an orbital shaker (New Brunswick Scientific, Edison, NJ, USA) at 120 rpm in a culture room maintained at 24  $\pm$  2°C under continuous illumination with cool white fluorescent tubes with a light intensity of 500 - 1000 lux. After 9 days, the cells were harvested and used as plant materials for subsequent studies.

##### *Effect of light intensity*

A cell biomass of 0.75 g obtained from the cell suspension stock, which was subcultured every 9 days in liquid MS medium + 0.25 mg/L BA + 0.5 mg/L NAA, was transferred into 100 mL Erlenmeyer flasks, each containing 20 mL liquid medium of the same composition. The pH of the medium was adjusted to 5.75 before autoclaving. Light intensities were measured using a lux meter with the light detector placed at the level of the liquid medium on the shaker. All prepared cell cultures were placed on the same shaker

(New Brunswick Scientific, Edison, NJ, USA), fitted with two fluorescence tubes from the top, supplying a range of light intensities up to 1200 lux. The light intensities were divided into four categories, i.e. 0 - 300 lux, 301- 600 lux, 601 - 900 lux and 901 - 1200 lux. The cells were harvested after 9 days of culture. The fresh cell biomass was determined using the digital balance (Denver Instrument XL-410, U.S.A.) and the dried cells were recorded after freeze drying. Pigment content and pigment production were determined as stated in the section below.

##### *Effect of continuous irradiance or continuous darkness*

To determine the effect of the photoperiod, a cell biomass of 0.75 g obtained from the cell culture stock was inoculated into each 100 mL Erlenmeyer flask containing 20 mL of cell proliferation medium. The cultures were divided into four groups. Each group was exposed to different photoperiods with light intensities maintained within the range of 301 - 600 lux. The four photoperiods were: 10 days of continuous exposure to illumination (L\_10d), 10 days of continuous darkness (D\_10d), 5 days of darkness followed by 5 days of exposure to illumination (D\_5d L\_5d) and 5 days of exposure to illumination followed by 5 days in darkness (L\_5d D\_5d). All the cultures were placed on the same shaker and maintained at the same temperature (24  $\pm$  2°C). The photoperiod effect was tested on cell biomass and anthocyanin accumulation after 9 days of culture.

##### *Effect of temperature*

A cell mass of 0.75 g obtained from the cell culture stock was inoculated into each 100 mL Erlenmeyer flask containing 20 mL cell proliferation medium. The pH of the medium was adjusted to 5.75 before autoclaving. The cell cultures were incubated at three different temperatures: 20  $\pm$  2°C, 26  $\pm$  2°C and 29  $\pm$  2°C. All the cultures were exposed to light intensities maintained within the range of 301 - 600 lux. The cell biomass yield, pigment

content and pigment production were determined after 9 days of culture.

#### *Effect of medium pH*

In order to find out the effect of medium pH on cell yield, pigment content and pigment production, a cell biomass of 0.75 g obtained from the cell stock was inoculated into each 100 mL Erlenmeyer flask containing 20 mL of the cell proliferation medium. The medium was adjusted to different pH before autoclaving: 4.25, 4.75, 5.25, 5.75, 6.25 and 6.75. All the cultures with different pH were placed under the light intensities maintained within the range of 301 - 600 lux. The cell biomass yield, pigment content and pigment production were determined after 9 days of culture.

#### *Quantification of pigment content and pigment production*

The total anthocyanins, represented by pigment content and pigment production of each culture, were quantified based on its colour (CV)/g-DCM or CV/flask, respectively (CV-Colour value; DCM- Dry Cell Mass; CV/Flask- colour value per flask). Freeze-dried cells of 80 mg were placed into a specimen bottle and 10 mL of acidified methanol (methanol + 1 % HCl v/v), the best extraction solvent, was added to the cells and stirred. The mixtures were covered and left in the refrigerator at 10°C for 24 hours. Absorbance of the supernatant of each sample was determined at wavelength 535 nm using the Pharmacia Novaspec II Rapid spectrophotometer against the blank which consisted of the solvent. Pigment content, CV/g-DCM, was adapted from Mori et al (1993) as follows:

Pigment content =  $0.1 \times OD_{535} \times$  dilution factor (CV/g-DCM)

Pigment production, CV/flask, was calculated by multiplying the pigment content with the respective mean dried cell mass obtained at the end of culture.

#### *Statistical analysis*

For each study, seven replicates were used for each factor (light intensity, photoperiod,

temperature and medium pH) and each study was carried out using a complete randomized design (CRD). Cell biomass yield, pigment content and pigment production were determined after 9 days of culture. The data were analyzed using a one-way ANOVA followed by Duncan's multiple range test for mean comparison at  $p = 0.05$ .

#### RESULTS AND DISCUSSION

Based on the growth index and anthocyanin content, a pigmented cell culture of *M. malabathricum* could be well maintained with an initial inoculum of 0.5 g of fresh cells inoculated into 20 mL of liquid MS medium + 0.25 mg/L BA + 0.5 mg/L NAA in 100 mL Erlenmeyer flasks and placed on a rotary shaker at 100 - 120 rpm. Most suspension-cultured plant cells fail to detach completely after division, instead they form multi-cellular aggregates (Meyer et al., 2002) and this includes the *M. malabathricum* cultures. During subculture, a sterilized stainless steel sieve with 850  $\mu\text{m}$  pore size was used to separate small cell aggregates from the bigger ones and only the smaller cell aggregates were used for the studies. This is because as cell aggregates enlarge, cells located in the interior of a large cell cluster are not uniformly exposed to nutrients, lighting, oxygen, and other micro-environmental factors, while the younger peripheral cells are. These local concentration gradients could alter cell proliferation, as well as secondary metabolite production.

Cell biomass yield and pigment production of cultures can be affected by several physical factors, including light intensity, photoperiod, temperature and initial medium pH. Experiments on each aspect were carried out to obtain the best condition for high cell biomass yield and pigmentation in cell suspension culture of *M. malabathricum*.

Light was found to be an important factor that affected biomass yield in cell suspension culture of *M. malabathricum*. It was also one of the most important factors in inducing and maintaining pigmentation

in the cells. Light intensities in the range of 301 - 900 lux stimulated growth of culture and lead to achievement of the highest fresh cell mass at the end of culture period. However, the highest pigment production was obtained from cultures exposed to a narrower range of light intensities i.e. 301 - 600 lux. Very high light intensities of 901 - 1200 lux reduced the biomass yield by 24 % and lowered pigment production by 87 %. Thus, light intensity had a stronger effect on pigment production than biomass yield. Incubation of cultures under light intensities of 301 - 600 lux, which produced a 10-fold increase in pigment production as compared to cultures exposed to light intensities of 0 - 300 lux, was identified as the most suitable for both cell growth and pigment production.

Our results revealed that cultures exposed to low light intensities (below 300 lux), increased cell biomass ( $0.85 \pm 0.25$  g fresh and  $0.06 \pm 0.02$  g dry). However, the cultures exposed to the light intensity between (301 - 600 lux) had the highest cell biomass ( $1.18 \pm 0.04$  g fresh and  $0.10 \pm 0.01$  g dry). Light intensity (above 901 lux) subsequently reduced cell biomass ( $0.90 \pm 0.10$  g fresh and  $0.07 \pm 0.01$  g dry). Exposure to medium a range of light intensities of 301 - 900 lux stimulated better cell growth as compared to extremely low or high light intensities (Fig. 1).

The effect of exposure to different light intensities was greater on pigment accumulation than on cell growth. At low light intensities (below 300 lux), the cultures produced pigment content of  $0.20 \pm 0.09$  CV/g-FCM and pigment production of  $0.19 \pm 0.13$  CV/flask. Medium light intensities (301 - 600 lux), induced higher accumulation of anthocyanins. The cultures exposed to these light intensities showed an increase in pigment content ( $1.62 \pm 0.14$  CV/g-FCM) and pigment production ( $1.91 \pm 0.14$  CV/flask). The cultures exposed to higher light intensities (601 - 900 lux) showed slight decrease in pigment content ( $1.20 \pm 0.52$  CV/g-FCM) and pigment production ( $1.38 \pm 0.62$  CV/flask) and the highest range of light intensities (above 901 lux) caused a drastic reduction in pigment content ( $0.29 \pm 0.10$  CV/g-FCM) and

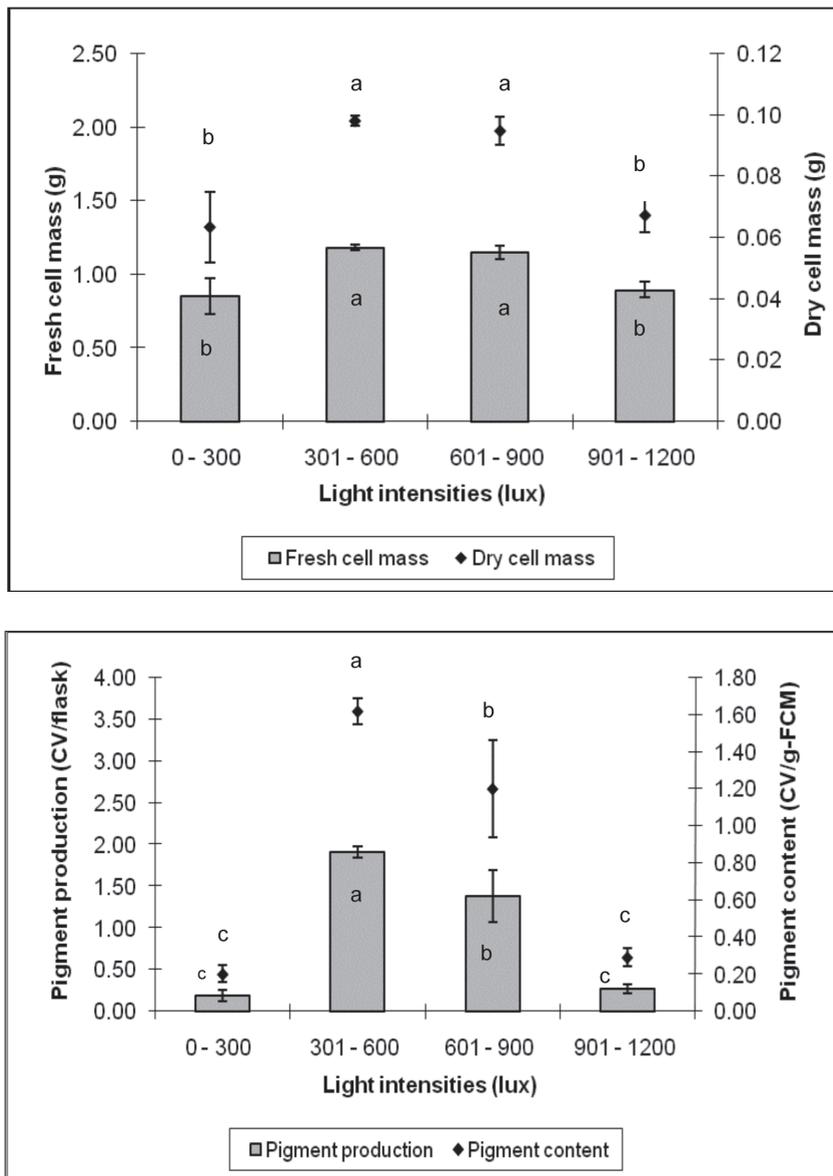
pigment production ( $0.27 \pm 0.11$  CV/flask). Exposure of cell cultures to extreme light intensities of either 0 - 300 lux or 901 - 1200 lux inhibited anthocyanin production (Fig. 1).

Experiments on the photoperiod further showed that continuous lighting for 10 days did not induce fresh cell biomass yield as much growth in darkness, which stimulated the highest anthocyanin production. On the other hand, continuous exposure to darkness throughout the cell cycle produced the highest biomass yield, but caused a major loss of pigmentation. Cultures that were exposed to light for 5 days followed by darkness for 5 days, or cultures that received the reverse treatment, produced fresh cell mass that were not significantly different. In terms of anthocyanin accumulation, cultures exposed to darkness, followed by exposure to light, produced better pigment content, suggesting that light exposure at the second half of the cell cycle was more crucial for the accumulation of pigment (Fig. 2). It is reported that light-enhanced production involved photosensory/photoregulation systems as the photoreceptor act as a switch to control the expression of specific genes involved in growth, developmental processes and secondary metabolism in plants (Jenkins et al., 1995; Ahmad et al., 1998; Kurata et al., 2000). In the *M. malabathricum* cell culture, the photoreceptor that control s the expression of genes involved in anthocyanin production could be seen to be triggered by exposure to 301 - 600 lux light intensities. After the process of anthocyanin biosyntheses was stimulated, it took the cells an average of 9 days to achieve maximum pigment content. Light has also been shown to induce anthocyanin biosynthesis in other cultures, such as *Camptotheca acuminata* (Pasqua et al. 2005). However, in some species, such as *Aralia cordata* (Sakamoto et al., 1993), *Fragaria ananassa* (Nakamura et al., 1999) and sweet potato (Konczak- Islam et al., 2000), cell cultures had been reported to produce anthocyanins in the dark.

Temperature has many effects on the mechanisms of metabolic regulation, permeability, nutritional needs, and the rate

of intracellular reactions in plant cell cultures (Zhang et al., 1997). Thus, changing the culture temperature may change the physiology and metabolism of cultured cells and subsequently affect growth and secondary metabolite production. A temperature range of 17 - 25°C is normally used for the induction of

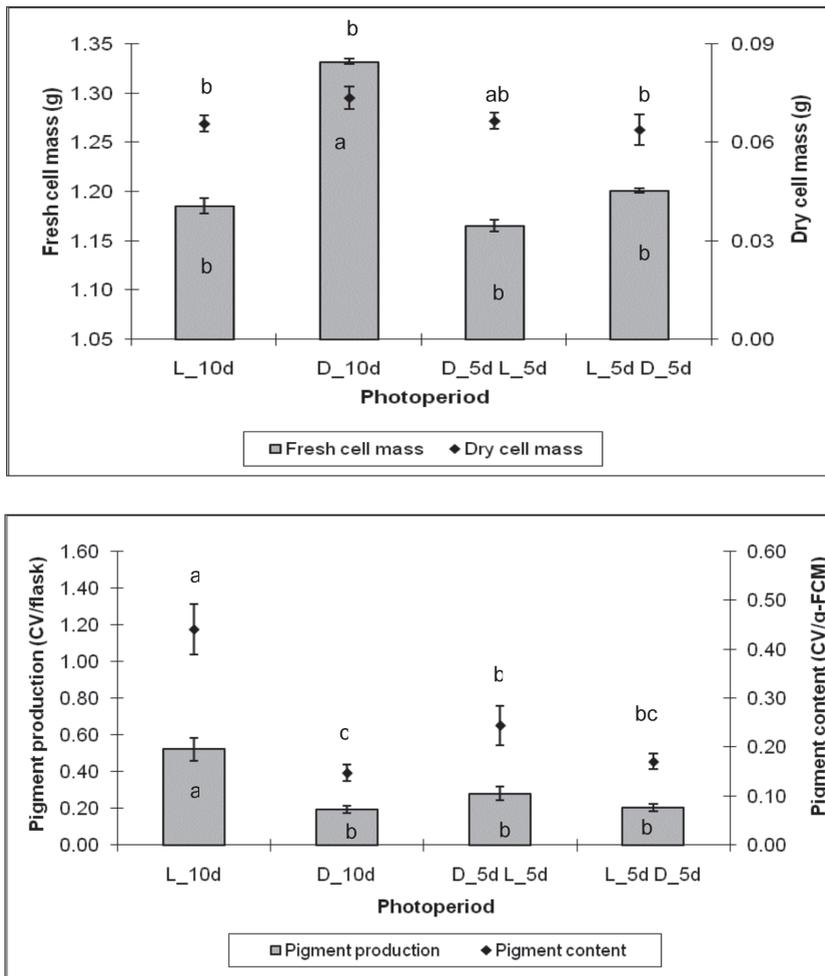
callus tissues and growth of cultured cells (Rao & Ravishankar, 2002). However, each plant species may favor a different temperature. Experimentation with cell suspension culture of *M. malabathricum* showed that temperature has a major effect on both cell biomass and anthocyanin accumulation. The cell cultures exposed to



**Fig. 1:** Effect of light intensities on fresh and dry cell mass (above); pigment content and pigment production (below) of cell suspension culture of *M. malabathricum* L. in liquid MS medium + 0.25 mg/L BA + 0.5 mg/L NAA (n = 7). Bars indicate standard deviations. Means for each parameter having the same letter were not significantly different (Duncan's multiple range test, p = 0.05).

the lower temperature range ( $20 \pm 2^\circ\text{C}$ ) showed the highest increase in cell biomass ( $2.49 \pm 0.55$  g fresh and  $0.12 \pm 0.02$  g dry) (Fig. 3). Besides producing the highest cell biomass, the cultures incubated at the lower temperature ( $20 \pm 2^\circ\text{C}$ ) also had the highest pigment production ( $1.04 \pm 0.17$  CV/flask) and pigment content ( $0.42 \pm 0.04$  CV/g-FCM). The cultures incubated at  $26 \pm 2^\circ\text{C}$  and  $29 \pm 2^\circ\text{C}$  had lower pigment production

( $0.20 - 0.24$  CV/per flask) and pigment content ( $0.21 - 0.30$  CV/g-FCM), respectively (Fig. 3). An increase of  $20^\circ\text{C}$  to  $26^\circ\text{C}$  caused a drop of 53 % in FCM and a drop of 77 % in pigment production. The effect of temperature could be linked to the activity of the enzymes involved in anthocyanin biosynthesis. Cell suspension cultures of *Daucus carota* showed the highest growth, as well as anthocyanin



**Fig. 2:** Effect of photoperiods on fresh and dry cell mass (above); pigment content and pigment production (below) of cell suspension cultures of *M. malabathricum* L. in liquid MS medium + 0.25 mg/L BA + 0.5 mg/L NAA. Bars indicate standard deviations. Means for each parameter having the same letter were not significantly different (Duncan's multiple range test,  $p = 0.05$ ).

Key:

L\_10d: 10 days of continuous illumination;

D\_10d: 10 days of continuous darkness;

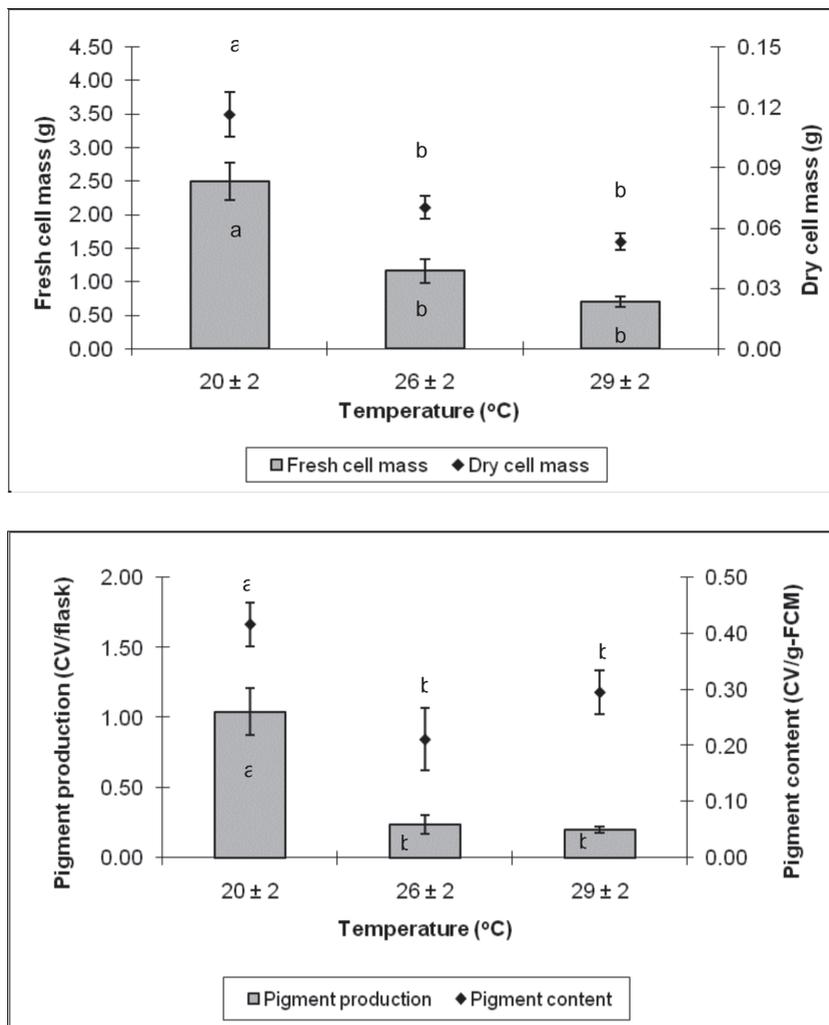
D\_5d L\_5d: 5 days of continuous darkness followed by 5 days of continuous light;

L\_5d D\_5d: 5 days of continuous illumination followed by 5 days of continuous darkness.

content, at 25 °C in an experiment with temperatures of 20 °C, 25 °C and 30 °C (Narayan et al., 2005). For strawberry cell culture, maximum anthocyanin content was obtained at 15 °C and it was about 13-fold higher than that obtained at 35 °C (Zhang et al., 1997). For suspended cultures of *Perilla frutescens*, anthocyanin production was remarkably reduced at the relatively high temperature of 28 °C, and 25 °C appeared to be optimal for the volumetric productivity of the pigment (Zhong & Yoshida, 1993).

For cell suspension culture of *M. malabathricum*, different initial medium pH that were tested (4.25, 4.75, 5.25, 5.75, 6.25 and 6.75) did not have much effect on the fresh and dry cell mass after 9 days of culture. The cell biomass yield with fresh cell mass of 1.4 - 1.8 g and dry cell mass of 0.11 - 0.12 g were not significantly different in all the tested pH ranges (Fig. 4).

Although the initial medium pH did not show significant effect on biomass yield, it affected anthocyanin accumulation in the



**Fig. 3:** Effect of temperature on fresh and dry cell mass (above); pigment content and pigment production (below) of cell suspension cultures of *M. malabathricum* L. in liquid MS medium + 0.25 mg/L BA + 0.5 mg/L NAA after 9 days of culture (n = 7).

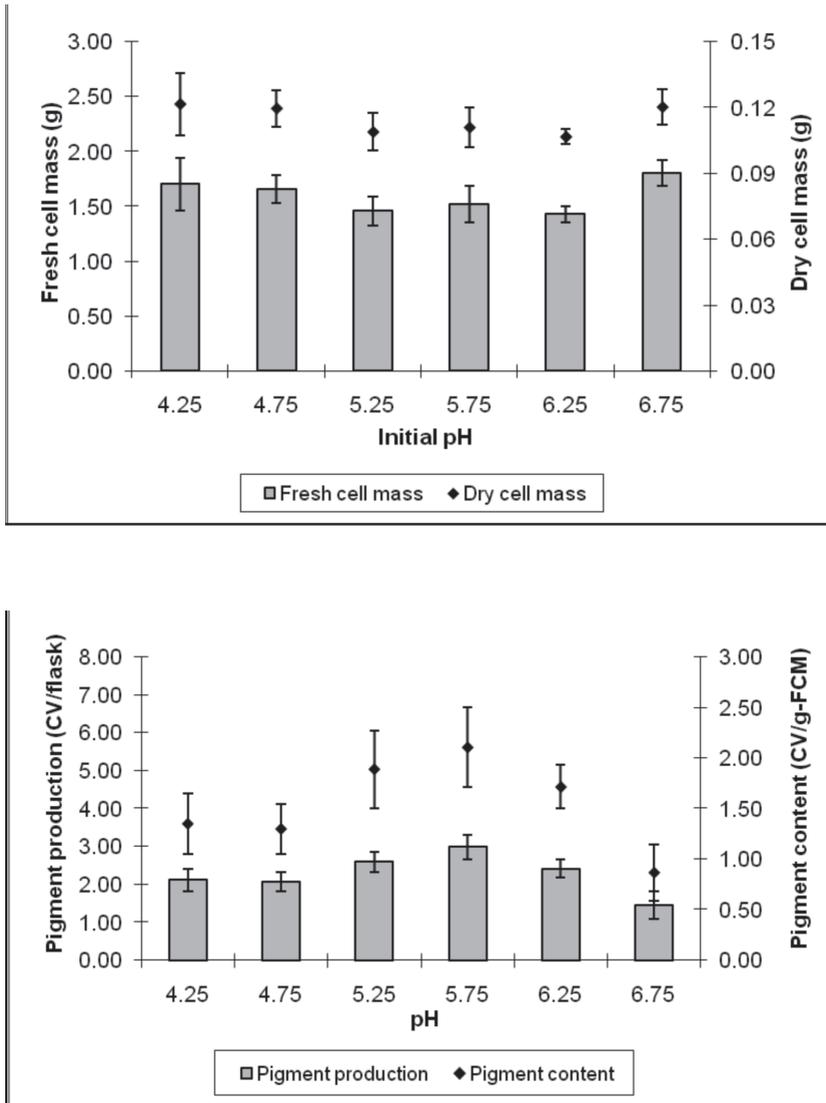
Bars indicate standard deviations.

Mean for each parameter having the same letter were not significantly different (Duncan’s multiple range test, p = 0.05).

cell cultures. Cell cultures with initial medium pH of 5.25 - 6.25 achieved higher anthocyanin accumulation, with pigment production of 2.42 - 2.99 CV/flask and pigment content of 1.71 - 2.11 CV/g-FCM. Both values were significantly different ( $p = 0.05$ ) from cultures in the more acidic or alkaline state. Anthocyanin accumulation with pigment production of 1.46 CV/flask and pigment content of 0.86 CV/g-FCM,

was the lowest for cell cultures in alkaline medium that supported the highest cell mass of  $1.80 \pm 0.24$  g (pH 6.75) (Fig. 4). Similar result was observed by Luthfi (2004), who found that the pH of the medium 5.75 (before autoclaving) produced the highest cell biomass and highest total alkaloids in cell suspension culture of *Eurycoma longifolia*.

The present study found that different



**Fig. 4:** Effect of medium pH on fresh and dry cell mass (above); pigment content and pigment production (below) of cell suspension cultures of *M. malabathricum* L. in liquid MS medium + 0.25 mg/L BA + 0.5 mg/L NAA after 9 days of culture (n = 7). Bars indicate standard deviations. Means for each parameter having the same letter were not significantly different (Duncan’s multiple range test,  $p = 0.05$ ).

factors, such as light intensity, irradiance, temperature and medium pH affect cell biomass yield and anthocyanin production in *M. malabathricum*. The exposure of cell cultures of *M. malabathricum* to the lower range (301 - 600 lux) of light intensities and continuous irradiance for 10 days could be used for higher pigment production. The lowest range of temperature i.e.,  $20 \pm 2^\circ\text{C}$  was found effective in production of the highest fresh cell mass, dry cell mass and pigment production. Cell cultures with initial medium pH of 5.25 - 6.25 showed higher anthocyanin accumulation in *M. malabathricum*.

#### CONCLUSION

To produce high cell mass and high anthocyanin content from *M. malabathricum* cells, the cell cultures have to be cultured in MS medium supplemented with  $0.25 \text{ mg dm}^{-3}$  benzyladenine (BA) and  $0.5 \text{ mg/L}$  NAA with pH adjusted to 5.75 and maintained at a temperature of  $20 \pm 2^\circ\text{C}$ , with exposure to continuous light, with light intensity in the range of 301 - 600 lux.

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