# Establishment of Cell Suspension Cultures for Plant Secondary Metabolites Study in *Barringtonia racemosa* L.

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**Abstract:** The present study was conducted to initiate and establish a homogeneous cell suspension culture system in *Barringtonia racemosa* from endosperm-derived friable calli which are useful for plant secondary metabolites study. Initiation and establishment of cell suspension cultures study were carried out by using different treatments of liquid media involving different concentrations and combinations of 2, 4-D (0.5 and 1.0 mg/L) and kinetin (0.75, 1.5 and 2.0 mg/L) plant hormones in MS (Murashige and Skoog's) medium. The difference in the treatments was formulated based on the reference treatments which produced the most optimum friable calli suitable for cell suspension cultures initiation. The treatment consisted of 1.0 mg/L 2, 4-D and 1.5 mg/L kinetin (treatment A) was found to be the most optimum treatment by considering timely mean dry cell biomass changes at every 5 days, interval with its maximum record of $16.32\pm0.72$ g/L. The distinctive phases of cell growth involving lag, exponential and declining phases had been identified. However, no lag phase were identified from the optimum treatment while other two treatments (1.0 mg/L 2,4-D + 2.0 mg/L kinetin (treatment B) and 0.5 mg/L 2,4-D+0.75 mg/L kinetin (treatment C)) underwent lag phase during first 10 days in culture. These were followed by exponential phases which were continued and achieved the peak of highest dry cell biomass records on day 50 (treatment A and C) and 55 (treatment B). Afterwards, all treatments had shown a declining progress in mean dry cell biomass records. The findings pertaining to cell growth phases are providing a useful information on plant cellular and molecular processes studies in this species and would further be utilised in research related to plant secondary metabolites production.

**Key words:** *Barringtonia racemosa* L., cell suspension culture, plant cell growth, plant secondary metabolites, exponential, concentrations

## INTRODUCTION

Plant cell culture is an important and widely used tool for basic studies on plant biochemistry and molecular biology (Mustafa *et al.*, 2011). The homogeneity of an *in vitro* cell population, the large availability of material, the high rate of cell growth and the good reproducibility of conditions make suspension-cultured cells suitable for the analysis of complex physiological processes at the cellular and molecular levels. In addition, the plant cell suspension cultures provide a valuable platform for the production of high-value secondary metabolites and other substances of commercial interest (Moscatiello *et al.*, 2013).

*Barringtonia racemosa* L. (*B. racemosa*) is a type of medicinal plant species native to East Africa, Pacific Islands and Southeast Asia (Orwa *et al.*, 2009). The medicinal values of this mangrove species have been acknowledged to be among the herbs of choice in various tribes around the world (Ong and Nordiana, 1999). Furthermore, the pharmacological properties of *B. racemosa* had been scientifically proven and recorded in a number of studies as reviewed by Osman *et al.* (2015). Due to significant medicinal values and wide distribution, *Barringtonia racemosa* L. has been acknowledged to be pharmacologically valuable and has been traditionally used in various tribes. However, this mangrove species had been classified as endangered in certain parts of the world. Considering its medicinal properties and threat of extinction, therefore, the application of plant tissue culture through cell suspension culture system is seen to be relevant to be applied in *B. racemosa*.

To date, the studies pertaining to *in vitro* culture establishment in this species are limited, hence, requires

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further development and optimization studies related to the area. In the present study, the cell suspension cultures of this medicinally valuable plant species were established from endosperm-derived friable calli. The screening and identification for the most optimum treatment for the establishment of cell suspension cultures were carried out in this study. The studies were conducted to obtain the optimum homogeneous cell suspension cultures and to identify the growth phases of the cultures which would be suitable for plant secondary metabolites studies to be further carried out in this species.

## MATERIALS AND METHODS

**Callus induction and proliferation:** The optimum treatment and protocol for callus induction process were carried out by referring to the previously documented research by Osman *et al.* (2016). The media for callus induction was prepared by using MS salt as the basal media supplemented with 1.0 mg/L 2,4-D (Sigma, US) and 1.5 mg/L kinetin (Sigma, US) plant hormones, solidified with 0.7% Gelrite agar (Duchefa, Netherland) and fortified with 3.0% sucrose (R&M Chemicals, UK). Endosperm was used as the explant and the cultures were left incubated at 25±2°C in the dark for 4 weeks before callus proliferation process was taking place.

After 4 weeks in culture for callus induction phase, the calli obtained were scraped and isolated from the explants and further subcultured onto new media of similar treatment (1.0 mg/L 2, 4-D and 1.5 mg/L kinetin). After 2 weeks, the proliferated calli were collected and transferred onto new media and ready to be cultured for cell suspension cultures initiation.

**Initiation and establishment of cell suspension cultures:** The optimum treatment used during callus induction was used as a reference treatment for the initiation of cell suspension cultures. Three different treatments were used to screen the most optimum treatment for cell suspension cultures initiation. The treatments used were as follows (Table 1).

Friable calli with the weight of approximately 2.5 g were cultured into 100 mL culture medium. Three replications were used and the flasks containing cultures were continuously shaken on shaking incubator (Daihan Scientific, Korea) at 100 rpm in complete darkness at 25±2°C. After 7 days, the cells were sieved to isolate the fine cells from the coarse, larger clumps of cell aggregates and maintained under similar culture conditions likewise before being sieved for cell suspension cultures establishment.

Table 1: Treatments used for initiation of cell suspension cultures

| Treatments | Concentration of 2,4-D+kinetin (mg/L) in MS media |
|---|---|
| A | 1.00+1.50 |
| B | 1.00+2.00 |
| C | 0.50+0.75 |

**Identification of cell growth kinetic:** Growth and development of cells in the suspension cultures were recorded by considering changes in the dry cell biomass of cell suspension cultures. The dry biomass were recorded at every 5 days interval for a duration of 60 days and the data gathered were plotted to form a growth curve of dry cell biomass (g/L) over time (day). Distinctive different phases were determined from the cell growth curve by taking into account the shape of the curve produced which reflected the timely changes in the cell growth. The optimum treatment was identified by considering the greatest dry cell biomass recorded amongst all the treatments tested.

**Microscopic observation of cell suspension cultures:** The cell suspension cultures were subjected to microscopic observation after 15 and 60 days in culture. Evans blue dye was used as staining agent at a concentration of 0.05%. Accurately aspirated 1000 µL of cell suspension cultures at the respective growth phases were taken and mixed with three drops of Evans blue dye 0.05%. The mixtures were incubated at room temperature (approximately 25-27°C). After 15 min of incubation, the mixtures were viewed under Bright Field (BF) microscopy mode by using inverted microscope (Olympus, Japan). The cellular morphological differences between those phases were observed and noted.

**Statistical analysis of results:** Three replications were used for each treatment. The results for treatment optimization were analysed by using IBM SPSS Statistics Version 17.0 and one-way ANOVA were applied. Post-hoc analysis of Tukey Test was used to analyse the differences in timely cell biomass records occurred between each treatment with the mean difference considered as significant at 0.05 level $p < 0.05$.

## RESULTS AND DISCUSSION

**Initiation of cell suspension culture:** Different treatments were found to produce almost similar onset of growth kinetic in each phase of cell growth (Fig. 1). Nevertheless, the optimum treatment had been determined in liquid MS medium consisted of 1.0 mg/L 2, 4-D and 1.5 mg/L kinetin (treatment A) by considering the records of mean dry cell biomass (g/L) throughout the observation period of 60 days. Early exponential phase had been identified to
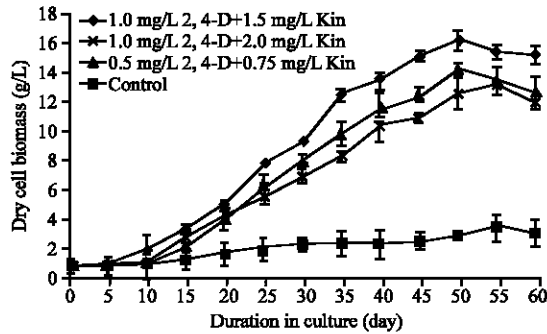
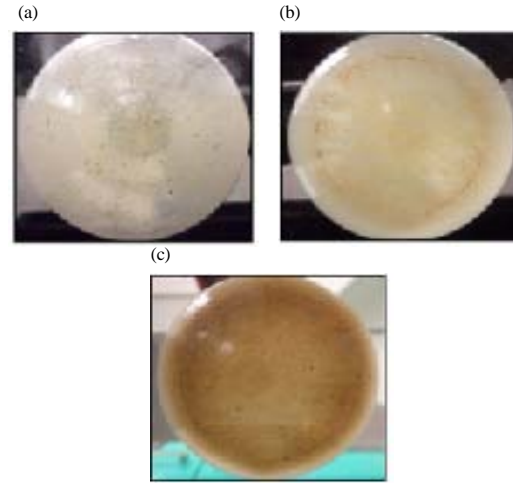Fig. 1: Growth of cell suspension cultures of *B. racemosa*



Fig. 2: Bottom view of flasks containing cell suspension cultures taken after; a) 15; b) 40 and c) 60 days of culture with the increase in turbidity over time

start as early as 5 days in culture in the optimum treatment while the rest of the treatments were considerably having lag phase during 5-10 days in culture. Meanwhile, the exponential phases were identified to have started after 10 days in culture in the treatment with the hormonal composition of 1.0 mg/L 2, 4-D+2.0 mg/L kinetin (treatment B) and 0.5 mg/L 2, 4-D+0.75 mg/L kinetin (treatment C).

The cultures achieved their highest mean dry cell biomass on day 50 in treatment A and C with the records of 16.32±0.72 g/L and 14.35±0.74 g/L, respectively. On the other hand, the highest mean dry cell biomass 13.33±0.33 g/L were recorded on day 55 in the culture of treatment B and the time onset (day 55) for maximum dry cell biomass to be achieved was similarly observed in control treatment 3.42±0.43 g/L. No distinctive stationary phase could be noted from all the treatments. Afterwards, all of the treatments were in the decline phase. In spite of having slight differences in the growth phases, the cell growth patterns of all the treatments tested were found to be almost similar whereby the dry cell biomass (g/L) were continuously increased until day 50-55 at which all treatments recorded the highest biomass and started to decline afterwards.

According to the statistical analysis, the effects of variations in media treatment on dry cell biomass were differed in their significance status according to the time in culture. However, generally there were no significant differences ($p > 0.05$) identified in the mean of dry cell biomass records during day 0 and 10 in culture while the rest showed significant difference ($p < 0.05$). Based on post-hoc analysis of Tukey HSD test, there were no significant differences identified in mean dry cell biomass only during day 0 and 10. There were only at certain points where the treatments were not significantly different to each other. Meanwhile, the rest (or the majority of the treatments) were observed to differ significantly. It was on day 5 that the treatment A was not differed significantly in the records of mean dry cell
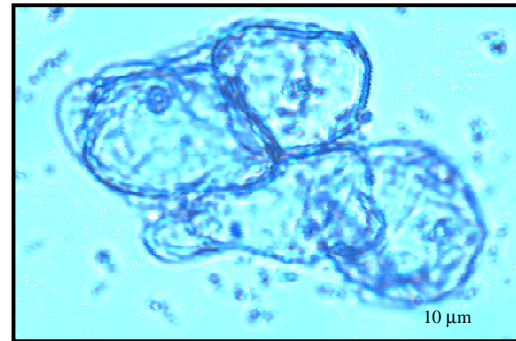


Fig. 3: Aggregation of rounded cells during early exponential phase (after 15 days) stained with Evans blue dye, viewed under bright-field microscopy at 100× magnification

biomass to treatment C and on day 15, treatment B and C were not differed significantly. Other than those mentioned, the effects of media treatments on dry cell biomass records were analysed to have significant difference ($p < 0.05$).

Upon microscopic viewing, cells during early exponential phase at the age of 15 days in culture were found to be mostly spherical (Fig. 2). Meanwhile, at a later stage of growth which is during decline phase at the age of 60 days in culture, most of the cells were found to be elongated (Fig. 3).

**Cell growth kinetic in cell suspension cultures:** Early exponential phase had been identified to start as early as 5 days of culture in treatment A while treatment B and C

were considerably having lag phase during first 10 days of culture. The lag phase in treatment A is therefore, assumed to occur earlier which was between 0-5 days of culture. Meanwhile, the exponential phases were identified to be started after 10 days of culture in treatment B and C. Exponential phase or also known as logarithmic phase is the phase where the cells grow exponentially and this phase was preceded by lag phase whereby in this phase the adaptation of cells to the provided growth conditions was taking place (Bulbuena *et al.*, 2009). No distinctive pattern of stationary phase was found from the growth curve in all, the treatments. Nevertheless, it was predicted to be achieved within a very short duration after maximum biomass was achieved in each treatment and started to decline afterwards. In this phase, the nutritional elements in the culture media were almost depleted and the growth of cells was almost ceased. Later, after 50 (treatment A and C) and 55 days (treatment B) in the cultures, the cells were in the decline phase which signified the death rate predominated and cell growth and development were completely ended. In spite of having slight differences in the onset of exponential phase, the cell growth patterns of all the treatments tested were found to be almost similar whereby the dry cell biomass were continuously increased until day 50 (treatment A and C) and 55 (treatment B) at which all treatments recorded the highest biomass and started to decline afterwards (Fig. 1).

The information gathered pertaining to the timely changes of cell growth in cell suspension cultures in *in vitro* dedifferentiated form is pertinent for various applications of phytoculture systems in research and commercial exploitation. In such a culture, a more convenient platform for plant cellular and molecular processes studies could be provided due to its simplified model system for the study of plants (Mustafa *et al.*, 2011). In addition, for the large scale cultures to be achieved in bioreactors for the production of phytochemicals, it is essential for the suspension cultures with a relatively homogeneous cell population to be initiated (Mustafa *et al.*, 2011; Crichton, 2014). The establishment of cell suspension cultures will allow rapid and uniform access of cells to nutrition, precursors, growth regulators and signal compounds (Mustafa *et al.*, 2011).

Distinctive growth phases identified from the cell suspension culture establishment are useful in providing information for the maintenance of cell suspension cultures to be carried out in this species. The information related to the changes of cell growth in a timely manner may serve as a clue for the subculturing procedure to be executed in future studies. Cells could not maintain their viability if they are left in stationary phase for a prolong

period due to limitation of nutrients and the cell number would reach the inhibiting level (Fleischer *et al.*, 1998). In order to maintain cellular viability, frequent subcultures at particular growth phase are required. Additionally, according to Heidarifar and Nayeri (2015), it is necessary to subculture regularly, since, the cultures tend to form clusters and aggregates which grow in clumps. For the subculturing purpose, the inoculum taken from stationary phase would usually result in an extended lag phase than those taken during linear phase of cell growth (Hall, 1991). However, in the current study, no subculturing procedures were carried out since the research was aimed to identify all phases of cell growth until it reaches the declining phase. Meanwhile, as for secondary metabolites production with the use of elicitors, the optimum time for the elicitor feeding is also phase-dependant. A close correlation between the growth of cultures and yield of products has been evidently demonstrated in various studies. Most of the documented research previously reported that the elicitors are best administered during late exponential (Ballica *et al.*, 1993; Jayaraman and Mohamed, 2015) or early stationary phases (Ramani and Jayabaskaran, 2008; Ahmed and Baiq, 2014).

**Visual observation of cell growth in cell suspension cultures:** The different stages of cell growth could also be noted by the differences in the colour intensity of the cell suspension cultures (Fig. 4). Turbidity of suspension cultures serves as a visual determinant that could be used to demonstrate the growth of cells and to evaluate cell viability since suspension culture turbidity is directly proportional to the cell density (Gaud *et al.*, 2008). In such observation, the opaqueness of the cultures would increase following cell density increase in a timely manner. In the present study, the consistency of the cultures
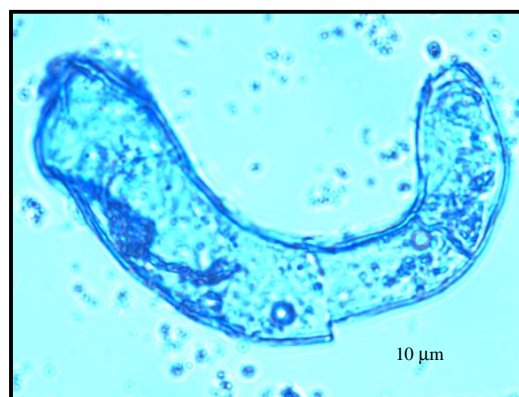


Fig. 4: Elongated cell during later stage of growth phase (after 60 days) stained with Evans blue dye, viewed under bright-field microscopy at 100× magnification

turned more viscous and turbid over times indicating cell growth has taken place. Finally, during decline phase, the cell suspension cultures were found to be obviously dark which indicated the death of cells had already occurred. The highest turbidity appearance during decline phase could be attributed to the release of organelles from the dead cells into the media (Fleischer *et al.*, 1998).

**Microscopic observation of cell suspension culture:** In suspension cultures, the cells are mostly form a few large aggregates and very rarely grow as single cell (Eibl and Eibl, 2009). Upon microscopic viewing from the observation of this study, despite having enormous range of cell morphology due to their asynchronous nature, the majority of cells in the suspension cultures showed distinctive shapes at different growth phases. Cells during early exponential phase were found to be mostly spherical and rounded (Fig. 2). Meanwhile, at a later stage of growth, mixed forms of cells were observed with the majority of them were found to be elongated (Fig. 3). The variety of cell morphologies shown by the cells in the suspension cultures indicated the occurrence of asynchronous division of cells (Warren, 1992).

Cell morphologies of suspension cultures are having a range of different shapes with the most frequently observed spherical and rod (sausage-like) shapes (Eibl and Eibl, 2009; Su, 2008). The morphological features of cells depend on several factors such as plant species, cell lines, growth stage and culture conditions (Kieran, 2001). In this study, the difference in shapes was identified to be mainly influenced by growth stage rather than culture conditions since the cell's shapes were found to differ in a timely manner. Suspension cultures also exhibit various degree of cell aggregation which consists of a mixture of mitotic and less mitotic cells (Su, 2008). It has been shown from the present findings that the later phase of growth in the suspension cultures were presented with elongated cell shapes and the changes of cells' morphologies were noted from mostly spherical to mostly elongated. The findings were in parallel to what had been stated by Su (2008), Eibl and Eibl (2009) in which plant cell elongation occurs after cell division ceases which could be understood to take place during late exponential phase or at the beginning of stationary phase.

Different morphological features of cells in suspension cultures had been observed by Mazarei *et al.* (2011) as well. However, the differences were noted from different types of physical characteristics of the cultures they established. Mazarei *et al.* (2011) found the sandy type culture consisted of large and elongated cells while the fine milky type contained small and rounded cells. On the other hand, the ultrafine type had shown various intermediate stages of the enlarged and small rounded cells.

**CONCLUSION**

A protocol to establish a homogeneous cell suspension culture in *B. racemosa* had been developed in the present study. Different phases of cell growth in cell suspension cultures of *B. racemosa* had been identified as well. The findings are useful for further scrutiny of plant secondary metabolites study in *B. racemosa* to be carried out.

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