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Factors affecting the establishment and growth of *Pogostemon cablin* cell suspension cultures

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ABSTRACT: Healthy and friable grown leaf-derived callus cultures of *Pogostemon cablin* were used to establish the cell suspension cultures in the shake flasks system. Manipulation of different culture conditions (light, carbon source, and inoculum size) were attempted in order to establish a rapid-growing and well-dispersed cell suspension cultures. Under the influences of different concentrations and combinations of carbon sources (sucrose, glucose and fructose), light conditions (continuous light, photoperiod and total darkness) and inoculum sizes (10, 15 and 20% (w/v)), increasing of biomass accumulation in *P. cablin* cell suspension cultures were observed. When the cells were cultured in MS medium containing different concentrations and combinations of carbon sources, the cell growth was the highest (0.29 g/20 mL of dry cell weight (DCW), or Growth Index, GI = 5.00) in the medium supplemented with sucrose (30 g/L). The results also revealed that 10% (w/v) inoculum size and culturing in the dark condition were the best conditions to produce a higher cells growth rate of 0.5 g/20 mL DCW (GI = 6.14). This is important for future studies on patchouli alcohol production that can be extracted from the cell suspension cultures of *P. cablin*.

KEYWORDS: *Pogostemon cablin*, callus cultures, cell suspension cultures, cell growth, plant cell culture

I. INTRODUCTION

Pogostemon cablin (or commonly known as patchouli plant) in various regions of the world serves as an important traditional medicine to treat diseases such as nausea, diarrhea, and headache [1]. It is one of the plant inhabitants in Asia and currently cultivated widely in Malaysia, Caribbean countries, Mauritius, West Africa, Philippines, China, and India [2]. Known by the name of 'Nilam' in Malaysia, the leaves of *P. cablin* were extracted to produce a crucial essential oil called patchouli oil [3]. Patchouli oil is a glutinous liquid and appears in between dark golden and light yellow color [4]. It consisted of compounds such as patchouli alcohol, α-bulnesene, pogostone, rosmarinic acid and eugenol [5]. This valuable oil can be used as a viral agent and possessed anti-microbial, anti-inflammatory and anti-tumor properties [6]. Apart from that, it has been applied in cosmetic as well as oral hygiene industries [1] and serves as a stress reliever and depression [7].

The fact that some plants are difficult to cultivate, grow slowly, and plant breeding takes several years has contributed to the search towards the application of plant cell culture technology as an alternative for the production of secondary metabolites. Plant biotechnology can be a potentially attractive alternative for the industrial production of these compounds. Past researches on *P. cablin* cell cultures have been conducted in order to determine the enhancement strategies suitable to increase the production of patchouli oil [8, 9]. However, very limited studies were found on the efforts to improve cell growth and subsequently increase the production of patchouli alcohol in cell suspension cultures through manipulation of physical and environmental aspects, as well as nutritional elements, which is perhaps the most fundamental approach to increase secondary metabolite production.

Carbohydrates, especially sucrose, are the important carbon and energy sources for most plant cell lines. It has been demonstrated that the initial carbon concentration can affect the growth rate and yield of secondary metabolites in plant cell cultures. Inoculum size is also an important factor for plant cell cultures, which can influence metabolite formation and hairy root growth as well as somatic embryogenesis [10, 11]. Nonetheless, light irradiation has a profound effect on the growth of cell cultures and production of plant metabolites including primary products such as enzymes, lipids and

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amino acids, and secondary products such as anthocyanins, flavonoids, terpenes and volatile oils [12]. Consequently, this research was conducted in order to study the factors that influence the growth of *P. cablin* cell suspension cultures in shake flask batch experiments in terms of nutritional (carbon source), physiological (inoculum size) and environmental (photoperiod) elements.

II. MATERIALS AND METHODS

A. Plant material and induction of callus cultures

Young leaves of *P. cablin* (Blanco) Benth were collected from the local plant garden center in Malaysia. The leaves were surface sterilized by shaking in mixtures of 20% (v/v) Clorox® with Tween-20 for 15 minutes, then rinsed thoroughly with sterile distilled water. Callus cultures were induced by placing the leaf explants (1 cm x 1 cm size) on Murashige and Skoog (MS) basal medium supplemented with 30 g/L sucrose, 8 g/L agar and 3 mg/L picloram. Cultures were maintained at 25 ± 2˚C in total darkness. The induced callus cultures were sub-cultured every 2-3 weeks on the same MS medium with a reduced concentration of picloram (1 mg/L).

B. Establishment of cell suspension cultures

The establishment of cell suspension culture started with the preparation of cell suspension medium. MS basal medium with Gamborg's vitamins (4.4 g/L) was mixed with picloram (1 mg/L). This medium was then designated as MS1P. The pH of the MS1P medium was adjusted to 5.8 using either 1 M NaOH or 0.5 M HCl prior to autoclaving at 15 psi and 121°C for 15 minutes. The initiation of cell suspension cultures was done by transferring 10% (w/v) of healthy and friable callus cultures into MS1P medium supplemented with 30 g/L sucrose (filter-sterilized via 0.2 µm Acrodisc syringe filter). The flask was then sealed with a cotton plug and maintained on a rotary shaker at 130 rpm and 25 ± 2˚C in total darkness. The cell suspension cultures were sub-cultured every 7 days by adding 60 ml fresh liquid medium until it was intense enough to be used as an inoculum for the next experiment.

C. Growth measurement

The growth of *P. cablin* was measured in terms of fresh cell weight, dry cell weight as well as the color of the cell suspension cultures. Fresh cell weight was determined by filtering the cell suspension cultures using Whatman filter paper No. 1, whereas the dry cell weight of the cell suspension cultures was determined after the cells were dried for 24 hours using freeze drier.

D. Effect of different carbon sources

In this study, the effect of three carbon sources (sucrose, glucose and fructose) on the growth of *P. cablin* cell suspension cultures were studied. Approximately 2 g of fresh cells (without medium) was transferred into 20 ml MS1P medium in 100 ml Erlenmeyer flask supplemented with various concentrations and combinations of carbohydrates as shown in Table 1. The shake flasks were then covered with a cotton plug and aluminum foil. The cell suspension cultures were shaken at 130 rpm, 25 ± 2˚C for 15 days in total darkness. Each experiment was repeated in duplicate to illustrate the trend of the cell growth. The samples were collected every three days. The fresh and dry cell weights were measured and recorded. To minimize differences in growth that may have been the results of variation in inoculum size, results are also presented as a Growth Index (GI):

$$GI = \frac{(Wt_{max} - Wt_{min})}{Wt_{min}}$$

where Wt_{max} and Wt_{min} are the maximum and minimum dry cell weight, respectively.

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Table 1: Different concentrations and combinations of carbon sources used in the study

Set	Carbohydrate Combinations
A	30 g/L Sucrose
B	30 g/L Glucose
C	30 g/L Fructose
D	10 g/L Glucose and 20 g/L Fructose
E	15 g/L Glucose and 15 g/L Fructose
F	20 g/L Glucose and 10 g/L Fructose
G	20 g/L Sucrose
H	60 g/L Sucrose
I	80 g/L Sucrose

E. Effect of different inoculum sizes

Different amount of inoculum (10, 15 and 20% (w/v)) was introduced into 20 mL of fresh MS1P medium in a 100 mL Erlenmeyer flask. The shake flasks were then placed on a rotary shaker at 130 rpm and the cell suspension cultures were grown at 25 ± 2˚C in the dark condition. The experiment was conducted for 15 days with a duplicate to illustrate the trend of the cell growth. All samples were collected every three days. The fresh and dry cell weights were measured and recorded.

F. Effect of different light conditions

Three different light conditions were introduced to *P. cablin* cell suspension cultures. The cultures were either exposed to continuous light, photoperiod (16 h light and 8 h dark) or maintained completely in the dark. The response to light was measured in terms of dry cell weight.

III. Results and Discussion

A. Effect of different carbon sources

Three different carbon sources (sucrose, glucose and fructose) with various concentrations and combinations from Table 1 were supplemented into MS1P medium to study the effect of carbon sources on the growth rate of *P. cablin* cell suspension culture. After 15 days of observation (Fig. 1), it was found that the cell cultures grew well in the medium supplemented with 30 g/L sucrose (Set A), reaching the highest growth index (GI = 5.00), followed by Set G (20 g/L sucrose) (GI = 3.20) and Set I (60 g/L sucrose) (GI = 3.14). The results are consistent with the nutrient requirement by most of the plant cell cultures [10-12]. However, Abdullah *et al*. reported that 3% and 5% of fructose and mannitol, respectively, were able to promote the growth of *Morinda elliptica* cell cultures [13], while fructose was the best carbon source for *Ficus deltoidea* cell suspension cultures [14]. Although *P. cablin* cell cultures preferred sucrose as the main carbon source, however at higher initial sucrose concentration (80 g/L, Set I, GI = 2.20), the cell growth was suppressed because of higher osmotic pressure. It was also noted that the cells could not utilize the combination of glucose and fructose in the medium as shown by the lowest GI in Set D-F. When glucose and fructose were supplemented individually in Set B and Set C, respectively, the cells managed to utilize them, although not as efficient as sucrose.

It is inevitable that sugar plays multiple roles in all aspects of plant life [15]. For example, carbon and energy were provided in the cell cultures by adding sugar [16]. Meanwhile, sugars also become a signal to vary stress reaction, growth and development of cell cultures [17]. Sucrose was the main transport sugar in plants and the major photosynthetic product. In addition, glucose and fructose produced in hydrolytic hexose by sucrose have affected the

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growth of the cell. This proves that in carbon allocation and sugar signal generation, sucrose plays the key regulatory role [17]. Furthermore, sucrose plays an important compound in which it can improve the production of secondary metabolites such as the production of paclitaxel in *Taxus yunnanensis* [11], anthocyanin in *Perilla frutescens* [12] and

Daucus carota cell suspension cultures [18], and anthraquinone in *Morinda elliptica* [19]. Moreover, the influence of sucrose medium in ginseng cells suspension cultures (*Panax* sp.) was very efficient to increase the production of ginseng saponin and ginseng polysaccharide as antitumor and immunological activities in medicinal technology [20].

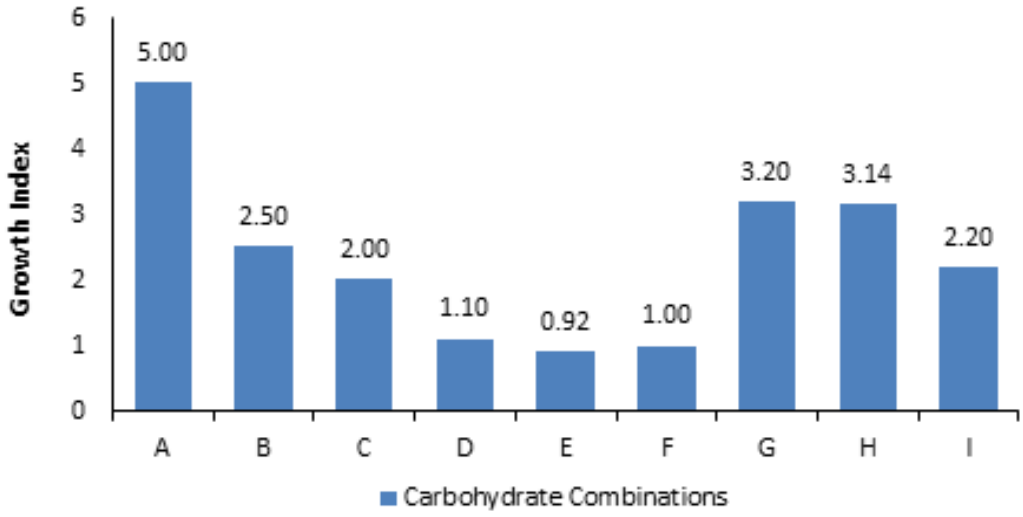

Fig. 1. Effect of different carbohydrate combinations on the growth of *P. cablin* cell suspension cultures. Growth was measured as an index. Each letter represents the combination of carbohydrates as listed in Table 1.

B. **Effect of different inoculum sizes**

Fig. 2 shows the biomass profile for different inoculum sizes. The highest biomass production was observed in 10% (w/v) inoculum size with 0.5 g/20 mL DCW (GI = 6.14) was obtained, followed by 20% and 15% (w/v) inoculum size, with the biomass production of 0.39 g/20 mL DCW (GI = 2.25) and 0.34 g/20 mL DCW (GI = 2.09), respectively. Treatment of 10% (w/v) inoculum size showed sigmoidal growth curve compared to 15% (w/v) and 20% (w/v).
The cell growth reached the exponential phase at day 6, after lag phase on day 0 to day 3 and entered stationary phase on day 12. However, the lag phase of 15% (w/v) and 20% (w/v) inoculum size treatment were longer which reached until day 6 and entered the exponential phase later, then reached the stationary phase on day 12. A long lag phase at higher inoculum size may be because of low concentration of some nutrients and growth factor due to the high rate of cells multiplication [21]. It was observed that the higher the inoculum size, the lower the growth rate and the biomass production. Similarly, inoculum size of 10% (w/v) was also the optimal condition for *Corydalis saxicola* Bunting cultures for biomass accretion and alkaloid production and the same inoculum size for the production of polyunsaturated fatty acids from *Marchantia polymorpha* cell suspension cultures [22].
It is, however, different for *Orthosiphon stamineus* cell suspension cultures, where 15% (w/v) inoculum was needed in order to obtain the optimum biomass accumulation [23]. In addition, the amount of inoculum density supplied also supports the modulated of metabolism of suspension cultures [24]. According to Ling *et al.*, a maximum inoculum size of 50% (w/v) was required in *F. deltoidea* cell suspension cultures [14]. Guo and Zhang also reported in their studies on ginger cell suspension culture that when the inoculum size was lower than 0.5% (w/v), low proliferation rate was observed while higher than 2.0% (w/v) lead to faster proliferation rate [25]. This indicates that plant cells required a critical minimum inoculum density for good cell proliferation as well as for secondary metabolite production.

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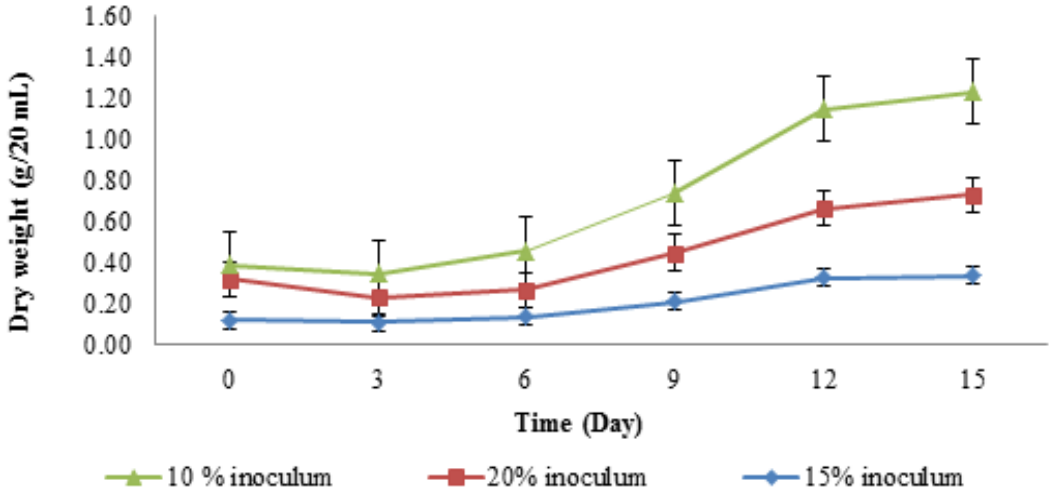

Fig. 2. Effect of inoculum size on the growth of *P. cablin* cell suspension cultures.

C. **Effect of different light conditions**

The *P. cablin* cell suspension cultures were grown in three different light conditions, i.e. under continuous light, photoperiod (16 h light and 8 h dark) and in total darkness. As shown in Fig. 3, the continuous light and photoperiod conditions were proven to be unfavorable to support good growth of *P. cablin* cell suspension cultures in which the dry weight of the cell suspension cultures decreased (± 0.18 g/20 mL DCW or GI = 3.0) towards the 15th days of the growth profile. On the other hand, cell suspension cultures that were grown in dark condition exhibited a very good growth rate, where the dry cell weight increased to 0.5 g/20 mL DCW (GI: 6.14) during the 15th days of the growth profile. The result of this study is in line with the research conducted by Behbahani *et al.* in which the growth profile of *Barringtonia racemosa* cell suspension culture was the best when grown in total darkness [26]. For *M. citrifolia* cell cultures, the cell growth and anthraquinone production were significantly improved when grown in the dark condition than under 500 lux light intensity [19].

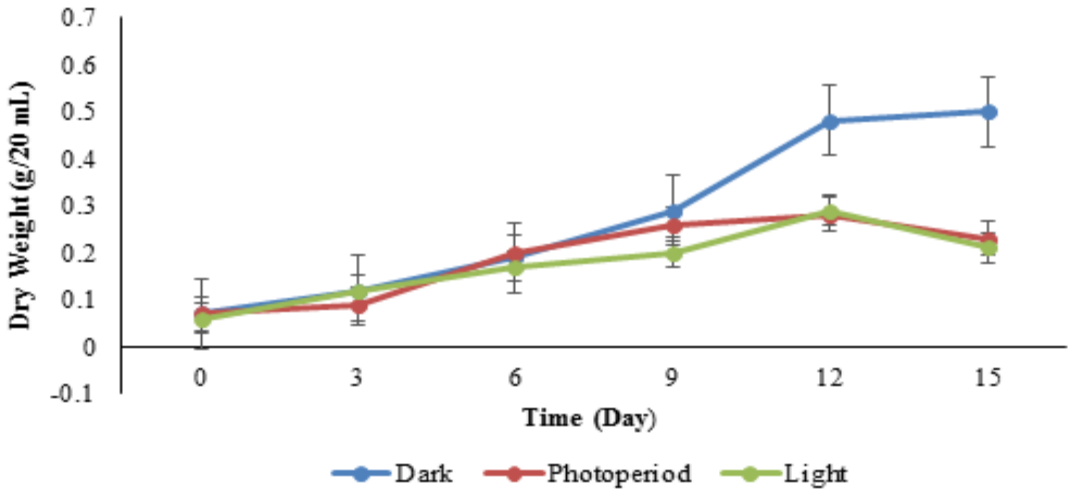

Fig. 3. Effect of different light condition on the growth rate of *P. cablin* cell suspension cultures.

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IV. CONCLUSION

The present study on *P. cablin* cell suspension cultures showed that the cell growth was influenced by carbon sources, inoculum sizes and photoperiod regimes. The best carbon source of 3% (w/v) sucrose was found suitable for the growth of *P. cablin* cell suspension cultures. Maximum biomass accumulation was obtained when 10% (w/v) inoculum size was used and the cells were grown in the dark condition. These results are useful for large scale cultivation of *P. cablin* cell suspension cultures for the production of patchouli alcohol.

V. ACKNOWLEDGEMENT

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