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Establishment and Growth Optimization For Callus and Cell Suspension Cultures of Pogostemon Cablin

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ESTABLISHMENT AND GROWTH OPTIMIZATION FOR CALLUS AND CELL SUSPENSION CULTURES OF *POGOSTEMON CABLIN*

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ABSTRAK

Dalam kajian ini, teknik kultur sel tumbuhan telah digunakan untuk mengkaji penghasilan minyak pati patchouli dalam kalus dan kultur ampaian. Kultur kalus telah dihasilkan daripada cebisan daun pokok *Pogostemon cablin* yang dipanggil eksplan. Pelbagai jenis, kepekatan pengatur pertumbuhan pokok (PPP) dan pelbagai keadaan kultur (cahaya, sumber karbon dan saiz inokulum) telah dikaji untuk menghasilkan pertumbuhan kultur kalus *P. cablin* yang cepat dan kultur ampaian sel yang menyerak sekata. Keadaan terbaik bagi pertumbuhan kultur kalus telah diperolehi dalam media asas MS yang mengandungi 1 mg/L pikloram, yang disenggarakan di dalam gelap selama 21 hari. Kultur kalus adalah sihat dengan ciri-ciri putih kekuningan dan berair. Bagaimanapun, eksplan yang dikulturkan menunjukkan respon negatif kepada media MS yang ditambah dengan 2, 4-D. Kultur kalus yang sihat, rapuh dan cepat membesar kemudiannya digunakan untuk menghasilkan kultur ampaian sel. Di bawah pengaruh pelbagai kepekatan dan kombinasi PPP serta cahaya, pertumbuhan kultur ampaian sel yang cepat telah diperhatikan dalam media MS yang ditambah dengan 1 mg/L picloram dan disenggarakan dalam gelap. Apabila sel ditumbuhkan dalam media yang mengandungi sumber karbon yang berbeza, pertumbuhan sel paling tinggi telah diperolehi dalam media yang ditambah dengan glukosa, sama ada secara individu (13.76 g/L) ataupun dalam kombinasi bersma fruktosa (12.06 g/L). Pada julat kepekatan sukrosa yang dikaji (2 – 8% (berat/isipadu)), didapati 6% (b/i) adalah yang terbaik untuk meningkatkan pertumbuhan sel (14.59 g/L). Keputusan ujikaji juga menunjukkan bahawa 10% (b/i) saiz inokulum adalah cukup untuk menghasilkan kadar pertumbuhan sel yang tinggi, dengan biomassa 24.95 g/L. Analysis GC-MS mengesahkan kehadiran minyak pati patchouli dalam ekstrak daun, tetapi ia tidak dapat dikesan dalam kedua-dua kultur kalus dan ampaian sel. Strategi untuk membentuk media untuk pertumbuhan dan penghasilan adalah penting untuk menggalakkan penghasilan minyak pati patchouli terutamanya dalam kultur sel ampaian.

ABSTRACT

In this study, plant cell culture technique was used in order to investigate the production of patchouli alcohol in callus and cell suspension cultures. The callus cultures were induced from the intact *Pogostemon cablin* leaves called explants. Different types, concentration of plant growth regulators (PGRs) and different culture conditions (light, carbon source and inoculum size) were studied in order to produce a rapid-growing callus cultures and well-dispersed cell suspension cultures of *P. cablin.* The best condition for callus cultures growth was obtained in the Murashige and Skoog (MS) basal medium containing 1 mg/L picloram which was maintained in the dark for 21 days. Callus cultures were healthy with white, yellowish and watery features. However, the cultured explants showed negative response to MS medium supplemented with 2, 4-D. The healthy, fast growing and friable callus cultures were then used to establish the cell suspension cultures. Under the influences of different concentrations and combinations of PGRs and light, rapid growth of cell suspension cultures were observed in the MS medium supplemented with 1 mg/L picloram and maintained in the dark. When the cells were grown in the medium containing different carbon sources, the highest cell growth was obtained in the medium supplemented with glucose, either individually (13.76 g/L) or in combination with fructose (12.06 g/L). At the range of sucrose concentration tested (2 – 8% (w/v)), 6% (w/v) was the best in enhancing the cell growth (14.59 g/L). The result also revealed that 10% (w/v) inoculum size was enough to produce a higher cells growth rate (24.95 g/L). The GC-MS analysis confirmed the presence of patchouli alcohol in the leaf extract, but none were detected in the callus or suspension cultures, respectively. A strategy to formulate growth and production medium is crucial to promote patchouli alcohol production especially in the cell suspension cultures.

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1.0 INTRODUCTION

1.1 *Pogostemon cablin* - Patchouli Alcohol – micropropagation (tissue culture), fragrance and medicinal purpose

Pogostemon cablin (Blanco) Benth (Syn. *Pogostemon* patchouli Hook) is part of the *Lamiaceae* family and is generally known as patchouli. It grows naturally in subtropical Himalaya, Far East and Southeast Asia. Besides that, *P. cablin* is also widely cultured in Malaysia, Indonesia, China and Brazil (Bunrathep *et al.*, 2006). Based on total manufacturer of patchouli oil, 550 tonne/year which is more than 80% comes from Indonesia. Indonesia becomes the major states that produce patchouli oil (Singh and Ganesha, 2009). Sesquiterpenes cytotoxic chalcones and anti- mutagenic flavones are also found in *P. cablin*. The traditional clinicians use *P. cablin* to treat the diseases such as nausea, diarrhea, headache and fever. This oil can be used as anti-cancer agent to treat disease such as anti-influenza A (H2N2) virus (Wu *et al.*, 2011) and also has other anti-microbial, anti-inflammatory, antibiotic, anti-microbial , anti-tumor properties (Kurniawan *et al.*, 2011) and in skin infections, dandruff and eczema plays as antifungal properties (Singh and Ganesha, 2009). It also has been applied in cosmetic and oral hygiene for instance perfumes and toothpaste flavour (Lu *et al.*, 2011). Although there are some studies that had reported on callus induction of *P. cablin,* however the investigation on secondary metabolite production in callus or suspension cultures and patchouli alcohol level is still very scarce and remain unfamiliar. From the previous study, a long period of time is needed to associate the metabolite difference in tissues by using morphogenic process (Santos *et al.*, 2011).

Some researchers have been reported on rapid regeneration of patchouli plant by direct organogenesis using nodal explants (Kumar and Chawla 2007); (Misra 1996). However, very limited literatures were found to report on the establishment of callus and cell cultures of *P. cablin* for the production of patchouli alcohol. Plant cell culture is one of the approaches available to provide large amounts and a stable supply of bioactive compounds especially the ones exhibiting antineoplastic activity, e.g. Taxol, vincristine and vinblastine. According to Lu *et al.* (2011), 1.5 % of essential oils and more than 50 % patchouli alcohol was found in *P. cablin*.

1.2 Plant cell culture technique

In recent years, interest in plant-derived pharmaceutical products and drugs has increased tremendously. For instance, the discoveries of antileukemic alkaloids vinblastine and vincristine from *Catharanthus roseus* (Cragg and Newman 2005), cytotoxic effects on human cancer cells from *Elaeagnus angustifolia* (Zeng *et al.*, 2011) and anticancer drug Taxol derived from *Taxus* sp. cell culture (Ramachandra Rao and Ravishankar, 2002) have drawn attention of the researchers from all over the world to explore the prospect of other medicinal plants for their therapeutic abilities.

Plants also have always been the most prominent sources of carbohydrates, proteins, lipids as well as vitamins and medicines for the mankind. Over the millennia, plants also emerged as the valuable sources of a vast array of secondary metabolites used as flavours, fragrances, pigments, food additives, agrochemicals and pharmaceuticals (Sasson *et al.*, 1992). Although most of the plants can be cultivated for the production of these fine chemicals, the levels are often very low in the plant, and hence the extracted products are usually very expensive. Some of the compounds are extracted from the wild plants and this method creates pressure on the natural resources (Kirakosyan *et al.*,2009).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. This can be shown from *Panax quinquefolium* cultivation, which involves quite complicated technique and can only be harvested after around 4 - 6 years of cultivation. Hence, it takes a long time to supply the plant source.

Based on the economic market demand, application of plant and cell culture technology in producing the ginsenoside saponins as its pharmacologically active metabolites could be cost-effective (Zhang *et al.*, 1996). Furthermore, plant cell culture technology has been recognized in the late 1960s as a promising way to produce a secondary metabolite of plants. Various techniques have been studied widely to improve secondary metabolite productivity. To extract secondary metabolite from the plant by using plant cell culture techniques, large-scale system using bioreactor should be used. Some of the benefits of this method are; 1) production of

secondary metabolite is under controlled, especially from natural condition, 2) avoid influence from microorganism and pest, and 3) control the production of secondary metabolite and cell growth with regulation of the medium condition (Mulabagal and Tsay, 2004). There are some techniques used in plant cell cultures technology involving the explants, callus and suspension cultures.

1.2.1 Callus cultures

Callus is a mass of undifferentiated cells and is the essential starting material in most plant cell culture systems. For the initiation of callus cultures, healthy young leaves were used as the explants because they generally produce callus more easily and more quickly than mature leaves (Allan, 1991). There are several parts of the plant that can be used for callus culture process which are floral bud, embryo stem, root, petioles, cotyledon, shoot tips and node. These are called vegetative organs. For more effective callus induction, young vegetative organ should be used. The most important parameter to get a successful continuing cell culture is the explant source (Biswas *et al.*, 2010).

1.2.2 Cell suspension cultures

Cell suspension cultures can be established when the callus cultures were transferred into the liquid medium containing necessary nutrients to support the growth and continuously shaken on the shaker to provide the cells with sufficient oxygen. To observe the growth process, the most important factor is the measurement of biomass in the cell suspension cultures. There are some methods used for collecting sample in cell suspension cultures which are by calculating the fresh weight and dry weight, packed cell volume, cell number and settled cell volume (Madhusudhan *et al.*, 1995).

1.3 Factors influencing the growth of plant cell cultures

Manipulation of the culture environment must be effective in increasing the growth of plant cell cultures and also product accumulation. The expression of many secondary metabolite pathways is easily altered by external factors such as nutrient levels, stress factors, light and growth regulators. Many of the constituents of plant cell culture media are important determinants of growth and accumulation of secondary metabolites. In view of this, it is essential to establish the healthy growth of callus and cell suspension cultures as the source for the production of useful secondary metabolites. Various factors that influence the growth of callus and cell suspension cultures have been studied by many researchers including nutrient levels, light and plant growth regulators.

1.3.1 Effect of carbon source

To synthesize macromolecules and other cell constituents, the main respiratory substrates are sugars. It is useful as a carbohydrate would generate the energy and metabolic intermediates to produce macromolecules and other cell constituents. Different types of sugar would initiate different responses and it would also affect the plant metabolism, growth and development. The most operative and common carbon sources to support plant cell culture's growth are sucrose and glucose, although other carbohydrate sources are often used. However, in general, sucrose appears to be the preferred carbon source for cultured plant cell systems. It would support high growth rates, biomass yield and production (Omar*et al.*, 2004). The concentration of carbon source supplied in the cultures also affects the production of secondary metabolite. The role of carbon source in the growth of plant cell cultures can be seen by the increase of alkaloid through *Catharanthus roseus*, rosmarinic acid in *Anchusa jicinalis* and *Coleus blumei*, carotenoid from carrot, nicotine by *Nicotiana tabacum*, improved shikonin formation from *Lithospermum erythrorhizon*, ajmalicine, serpentine, and tryptamine in *Catharanthus roseus* cell cultures and Taxol in cell suspension cultures of *Taxus cuspidata* (Zhong and Yoshida 1995); (Omar *et al.*, 2004); (Zhang *et al.*, 1996).

1.3.2 Effect of plant growth regulator

The hormonal plant growth regulators (PGRs) usually have profound effects on both cell growth and secondary metabolite production (Franklin and Dixon, 1994). The type and concentration of auxin or cytokinin or the auxin/cytokinin ratio alters dramatically both the growth and the product formation in cultured plant cells (Mantell and Smith, 1984). Dichlorophenoxyacetic acid (2, 4-D) is the most widely used synthetic auxin, especially for gramineous species. 2, 4-D and naphthaleneacetic acid (NAA) have largely replaced the naturally occurring auxin, acetic acid (IAA) in cell culture media, as the latter is readily oxidized by plant cell. 6-Benzylaminopurine (BAP) and kinetin (KN) are the most commonly used cytokinin. It was established that 2, 4-D has stimulated the saponin synthesis from ginseng calli although indole-acetic acid (IAA) was constrained (Zhang*et al.*, 1996). By alteration of PGRs concentration, the growth of callus cultures of *Cupressus lusitanica* (Yamada *et al.*, 2003), *Orthosiphon stamineus* (Wai-Leng and Lai-Keng, 2004) and *Abrus precatorius L.* (Rahman *et al.*, 2012) were effectively improved.

1.3.3 Effect of light irradiation

One of the important physical factors to produce the secondary metabolites from plant cell cultures technique is light. Various studies presented that it may stop or encourage the production (Zhao *et al.*, 2001). Light also plays an important role in these studies and has affected the production of secondary metabolites of nicotine and shikonin. However, light has no influence on the biosynthesis of anthraquinone and ubiquinone metabolites (Liu *et al.*, 2002).

1.3.4 Effect of inoculum size

Another factor that is important in the establishment of *P. cablin* cell suspension cultures is the inoculum size or cell density. It would also affect the production of secondary metabolite (i.e. patchouli alcohol) (Zhang *et al.*, 2002). According to Yoshihiro and Atsushi (1985), the importance of this factor is to relate between the cell with each other and cell to medium interaction in cell growth. Besides that, the inoculum size would also affect the enzymes

interaction in growth cultures. For instance, inoculum density has affected the stimulation of enzymes participated in the common phenylpropanoid metabolism once cells were conveyed to the new medium. The terms of 'transfer effect' or 'dilution effect' can be described in these situations. It is also found that phenylalanine ammonia-lyse production decreased by increasing the inoculum size. Similar effect was also observed during the establishment of the *Saussurea medusa* cell suspension cultures. The smallest inoculum size is useful to continue the growth of cultures (i.e. subculture of cell suspension after 2-3 weeks of growth, based on the growth rate of the cells) (Zhao *et al.*, 2001). Moreover, production of anthocyanin and ginseng saponin by *Perilla frutescens*, polysaccharide from *Panax notoginseng*, increased of Taxol production and cell growth from *Taxus chinensis* suspension cultures were also impressively caused by the inoculum size (Wang *et al.*, 1997).

1.4 Enhancement strategies of secondary metabolite production

Various enhancement strategies can be applied to the systems to achieve significant amount of the targeted products. Although the capability of plants to produce such effective and valuable products has been proved elsewhere, the undifferentiated tissue cells, i.e.: callus cultures produce limited amounts of the desired products without the use of enhancement strategies. Such strategies including addition of precursor and elicitation in plant cell systems, cell immobilization, media optimization and others have been successfully implemented (Roberts and Shuler, 1997).

1.4.1 Precursor feeding

Precursor feeding is one of the popular approaches to increase secondary metabolite production in plant cell cultures. It is based on the concept that when the intermediate compound is supplied in or at the beginning of a secondary metabolite biosynthetic route, the chance to increase the yield of the final product is high. Attempts to induce or increase the production of plant secondary metabolites, by supplying precursor or intermediate compounds, have been effective in many cases. For instance, in cell suspension culture media, amino acids have been added to produce tropane alkaloids and indole alkaloids. The addition of phenylalanine as the precursor did not only improves the production of rosmarinic acid in

Salvia officinalis cell suspension cultures, but also reduced the production time. Phenylalanine is also the precursor of the *N*-benzoylphenylisoserine side chain of Taxol, and supplementation of *Taxus cuspidata* cultures with phenylalanine has resulted in increased yields of Taxol (Fett-Neto *et al.*, 1994; Fett-Neto *et al.*, 1993). Ferulic acid was also found to increase vanillin production when this compound was added in *Vanilla planifolia* cultures. (Mulabagal and Tsay, 2004).

1.4.2 Elicitation

Secondary metabolite(s) produced by plants in nature is one of the defense mechanism against attack by pathogens. Plants have been found to elicit the same response as the pathogen itself when challenged by compounds of pathogenic origin (elicitors). Elicitors are indicators in activating the development of secondary metabolites. It also plays as a plant protection from pathogens. Biotic and abiotic elicitors are to encourage the production of secondary metabolite in plant cell cultures by decreasing the time to get high concentration of product and increased culture volumes (Mulabagal and Tsay 2004): Elicitors can be either biotic (glucan polymers, glycoproteins, low molecular organic acids, or fungal cell materials) or abiotic (UV radiation, salts of heavy metals and various chemicals) (DiCosmo and Misawa, 1985).

2.0 MATERIALS AND METHODS

2.1 Introduction

This section consists of four important parts that has been explored to accomplish the ultimate aim of this studies which are plant cell cultures technique, preparation of cultures, experimental techniques and analytical procedures. The essential condition applied for this *P. cablin* cell cultures were presented below:

- Medium was sterilized by autoclaving at 120°C, 1 bar and 15 minutes.
- All process for cell cultures was performed under laminar flow hood.
- The cultures were incubated at 23°C in suitable light condition (total darkness, photoperiod and continuous light).
- The cell suspension cultures were continuously shaken on gyratory shake at 130 rpm.

2.2 Plant cell cultures techniques

2.2.1. Source of explant

The main material for this study was the leaves of *P. cablin*. The plants were obtained from local plant garden center. The leaves were used as the explants (piece of plant tissues) for callus cultures induction. The *P. cablin* tree is shown in Figure 2.1.

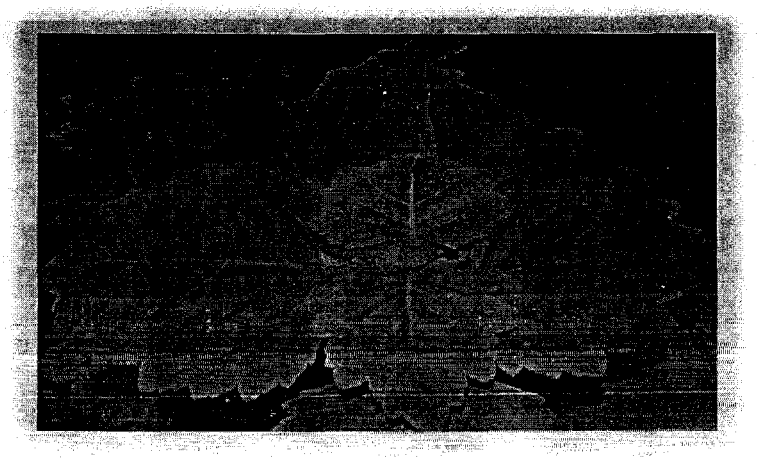

Figure 2.1: *Pogostemon cablin* tree

2.2.2 Chemicals

The chemicals used in this study were procured from Sigma-Aldrich and other companies as listed in the Appendix A. The plant growth regulators stock solution (Appendix B) were prepared earlier and stored in the refrigerator at 4°C. The solutions were regularly inspected and were discarded if any precipitation occurs. The filtrate samples were stored in the freezer at -20 °C for further analysis.

2.2.3 Culture media preparation

2.2.3.1 Solid medium

Three types of solid media were prepared in this study, based on the experiments to be carried out:

a) Initiation of callus cultures
b) Growth and maintenance of callus cultures
c) Effect of plant growth regulators on the growth of callus cultures

A liter of solid medium was prepared. The basic stock solutions were added into a 2 liters beaker which was filled up with 500 ml distilled water. Then, the mixtures were mixed by using the magnetic stirrer. After that, sucrose was added and stirred until it was completely dissolved. The solution was then added with distilled water until the volume reached 950 ml. At that time, the pH was adjusted at range 5.8 by using 1 M NaOH and 0.5 M HCl. Next, the medium was transferred into 1 liter volumetric flask and the distilled water was refilled until reached the 1 liter volume. The medium was transferred back into the beaker and fully mixed.

Agar was supplemented adequately as the solidifying agent into the medium. The stirring process was continued to ensure that the agar was absolutely dissolved in the medium. Afterward, the medium was divided into two 500 ml bottles proceeding to sterilization at 121 °C, 1 bar for 15 minutes. Once the autoclaving process has been done, the sterilized medium was placed in the laminar flow hood to allow the temperature to reach the room temperature. Subsequently, the medium was poured aseptically into the plastic sterile disposal petri dishes

19 x 55 mm and let it solidified. To avoid any contamination, evaporation of water and condensation occur on the top, these petri dishes were set aside in a dry place and overturned.

For the preparation of growth and maintenance culture media, the same procedure was used excluding some chemicals as summarized in Appendix C as a C1 and C2.

2.2.3.2 Suspension medium

Chemicals used in this preparation of 1 liter suspension medium were presented in Appendix C as C3. Murashige and Skoog (MS) basal medium with Gamborg's vitamins was dissolved in 500 ml distilled water in a 2 liter beaker. Then, the medium was stirred by using magnetic stirrer. Meanwhile, the picloram stock solution was added and mixed together. The pH medium then was regulated at 5.8 using 1 M NaOH or 0.5 M HCl. The solution was transferred to 1 liter volumetric flask and the absolute volume was adjusted to 1 liter. After that, the medium was divided into 500 ml each bottle. Next, the medium was sterilized with condition of 121 ℃, 15 minutes at 1 bar. Later than, sucrose was filtered sterilized via 0.2 µm Acrodisc syringe filter and was added into the medium that was cooled down at room temperature. To avoid from any contamination, the solution was kept at dry place for further experimentation.

2.3 Preparation of cultures

2.3.1 Callus cultures

2.3.1.1 Initiation of callus cultures from explants

There are several parts of the plant that can be used for callus culture process which are floral bud, embryo stem, root, petioles, cotyledon, shoot tips and node. This is called vegetative organs. For more effective, young vegetative organs should be used (Biswas *et al.*, 2010).

The most important parameter for successful continuing cell culture is explants source. Firstly, the leaves were cleaned by washing under running tap water to eliminate any large particle or dust and were dried on tissue papers. The intact surface of the dried leaves was wiped gently using cotton which was soaked with pure ethanol. Afterward, the leaves were

surface sterilized by immersing them in 20 % (v/v) Clorox solution with the addition of three drops of Tween 20 for 15 minutes under nonstop shaking. The leaves were then rinsed in sterilized distilled water under aseptic conditions for at least five times to remove any traces of Clorox. Subsequently, the leaves were cut into small squares (1cm x 1cm) by using the sterile scalpel. Before that, the cut surface were eliminated which were scratched during sterilization process.

Then, the explants were transferred aseptically, i.e. five-piece explants were arranged onto the solid media in the petri dishes. These petri dishes were sealed with parafilm to prevent dehydration of the media or some contamination. This medium is designated as MS3. The explants were incubated in the dark at 23°C until the callus cultures were induced after 7-10 days of incubation and frequently observed to prevent from any contamination. The contaminated explants were taken out to prevent the growth of fungal or bacteria on other explants.

2.3.1.2 Maintenance of callus cultures

The friable callus initiated from the explants was then detached from the explants and was transferred into the new initiation solid media. This procedure was done at least twice to ensure that the callus growth was sufficient enough before they were transferred into the maintenance medium, which contain different plant growth regulators to support their growth. Subsequently, the friable callus was sub-cultured into callus maintenance solid media by using sterile forceps. The callus was arranged about 4-5 portions with a size of small pies. All the process was done in the laminar flow hood. The callus was incubated in the dark at 23 ± 1°C. The callus was sub-cultured after 21 days depending on the growth rate.

2.3.2 Initiation of cell suspension cultures

The friable and healthy callus was chosen for the initiation of suspension cultures process. These callus cultures were sub-cultured for at least five times before they could be used to initiate the suspension cultures. About 10% (w/v) of friable callus (10 g) was transferred into

100 ml suspension medium in a 500 ml sterilized shake flask. To evade any contamination, the flask was covered tightly with the cotton and aluminum foil. Then, the flasks were shaken at 130 rpm continuously for 3 weeks under dark condition at 23°C. The cell suspension cultures were sub-cultured every 7 days by adding 60 ml fresh liquid medium until it was intensed enough and can be used as the inoculum for the experimentations. Regular inspection was done as mentioned in callus maintenance part. The diagram of establishment of callus and cell suspension cultures was shown in Figure 2.2.

2.4 Experimental techniques

2.4.1 Callus cultures

The general technique to study the effect of plant growth regulators (PGRs) and light irradiation on callus cultures was described below:

a) The callus was sub-cultured in MS solid medium containing different combinations of PGRs (Table 2.1) (i.e.: 2, 4-D: Dichlorophenoxyacetic acid, P: Picloram, BAP: 6-Benzylaminopurine, NAA-1: Naphthaleneacetic acid) in the jar bottle.
b) The size of callus was 2 cm in diameter.
c) The cultures were cultivated under 3 conditions which were in darkness, photoperiod and continuous light at 23°C for 30 days.
d) The observation of color, formation of callus size and types of callus induced was recorded.

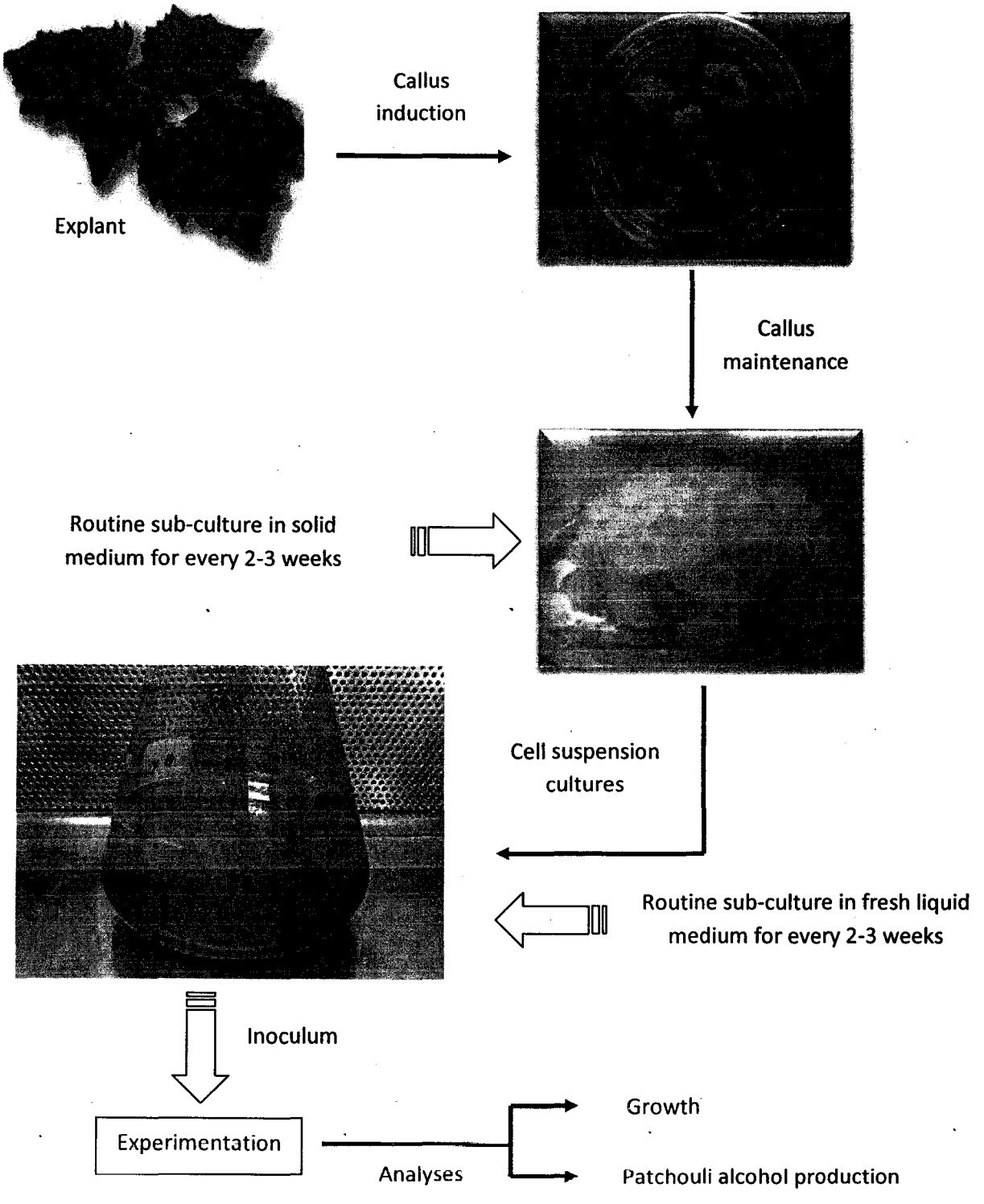

Figure 2.2: Schematic diagram for initiation of callus and suspension culture

Table 2.1: Combination of PGRs used in the study for the growth of *P. cablin* callus cultures

Media	Plant Growth Regulator (mg/L)		
	P	NAA	2,4-D
MS1	1	-	-
MS2	2	1	-
MS3	3	-	-
MS4	1	0.25	-
MS5	1	0.75	-
MS6	1	-	0.25
MS7	1	-	0.75
MS8	2	-	-
MS9	3	1	-
MS10	-	1	-
MS11	1	1	-
MS12	1	-	1

2.4.2 Suspension cultures

The cell suspension cultures were sub-cultured to study the effects of light irradiation, plant growth regulator and carbon source and inoculum size. Generally, after two weeks of sub-culture, about 2 g of cells without medium was transferred into 20 ml liquid media in 100 ml volumetric flask. The shake flask was covered with cotton and aluminum foil and shaking at 130 rpm, 23°C for 15 days. The experiment was conducted in 15 days to illustrate the trend of the cell growth. The samples was collected and measured every three days. The fresh weigh and dry weight was measured.

2.4.2.1 Effect of plant growth regulators

The effect of PGRs was studied by varying the concentration and combination of NAA, 2, 4-D, P and BAP. The combination of PGRs used is shown in Table 2.2.

Table 2.2: Combination of PGRs used in the study for the growth of *P. cablin* cell suspension cultures

Medium and light condition			Concentration of PGRs (mg/L)
Dark	Photoperiod	Continuous light	
MD1	MP1	MC1	1 P
MD2	MP2	MC2	1 BAP
MD3	MP3	MC3	1 NAA
MD4	MP4	MC4	1 2,4-D
MD5	MP5	MC5	1 2,4-D + 1 BAP
MD6	MP6	MC6	1 2, 4-D + 0.5 BAP

2.4.2.2 Effect of light condition

Three different light conditions were studied in combination with PGRs treatment for *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 irradiation was measured in terms of cell dry weight.

2.4.2.3 Effect of carbon source

To study the effect of carbon source on the growth of *P. cablin* cell suspension cultures, the MS liquid medium was supplemented with different concentration of sucrose, fructose and glucose (as given in Table 2.3). The suspension cultures were incubated at the same condition but in darkness. The cells were harvested every 3 days interval for 15 days and the dry weight were measured and recorded.

Table 2.3: Different combinations of carbon sources used in the study

SET	CARBOHYDRATE COMBINATION
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 (G2F4)
E	15 g/L glucose and 15 g/L fructose (G3F3)
F	20 g/L glucose and 10 g/L fructose (G4F2)
G	20 g/L Sucrose
H	60 g/L sucrose
I	80 g/ L sucrose

2.4.2.4 Effect of inoculum size

The effect of inoculum size on the growth of *P. cablin* cell suspension cultures were also carried out. Different amount of inoculum (10, 15 and 20% (w/v)) was introduced into 20 mL of fresh MS medium in a 100 mL Erlenmeyer flask. The flasks were then placed on rotary shaker at 130 rpm the cell suspension cultures were grown in the dark. The cells were harvested every 3 days interval for 15 days and the dry weight were measured and recorded.

2.5 Analytical procedures

2.5.1 Growth measurements in suspension cultures

The growth of callus cultures took a long period of time compared to cell suspension cultures. Sequentially, the fresh weight and dry weight mass from batch flask were measured to characterize the growth of the *P. cablin* cell suspension cultures. The empty Whatman No. 1 filter paper was weighed. Then, 20 ml of the flasks cultured was harvested through filter paper and weighed as a fresh weight. The filtrate was filter again. The supernatant was centrifuged

and the pH was measured before stored in the freezer at -20 °C for further sugar and product analyses. The dry weight was measured after the cells were dried for 24 h at 75°C in the oven. The dry weight was measured after it reached a constant weight.

2.5.2 Analysis of sugars

The residual sugar contents in *P. cablin* cell-free suspension medium were analyzed by using the High-Performance Liquid Chromatography (HPLC) (Shimadzu/ LC10AT-Vp) using the Hypersil APS-2 µm column (250 mm x 4.6 mm, 5µ particle size) with detector refractive index detector and mobile phase of acetonitrile: deionized water (80:20). The sample (filtrate) was filtered via 0.2 µm Supor membrane filter, Acrodisc 25 mm syringe filter. The mobile phase was conceded through the column with 0.5 ml/min of flow rate and the samples were alienated and detected by HPLC system. The computer integrated with the HPLC system was used to visualize the chromatogram analysis and store the results. Figure 2.3 represents the overall process during HPLC analysis of the residual sugars.

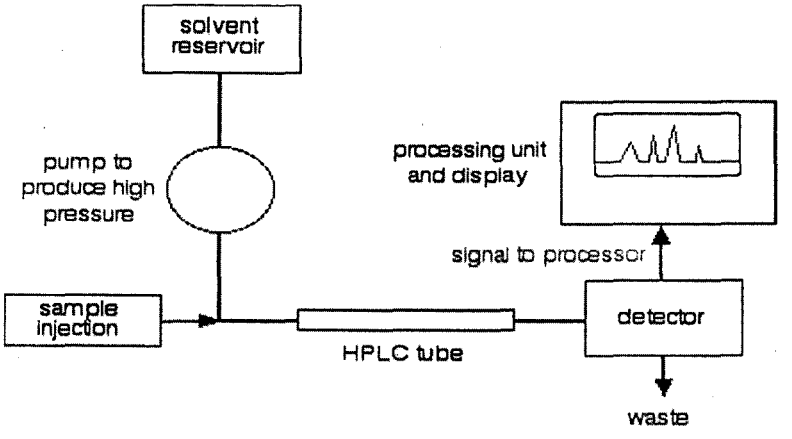

Figure 2.3: A flow scheme for HPLC analysis (http://www.chemguide.co.uk/analysis/chromatography/hplc.html)

Five different concentrations of sucrose, fructose and glucose ranging from 10, 20, 30, 50 and 100 g/L were prepared to construct the calibration curve before the analysis the residual content of the sugar can be conducted. The residual concentration of sucrose, fructose and glucose in the samples was calculated by using the counted peak area of the samples with the standard curves. The retention times for the sugars are summarized in Table 2.4 and the chromatogram is displayed in Figure 2.4.

Table 2.4: The retention time for each sugars standard

Standard No.	Compound	Retention time (min)
1	Fructose	8.058
2	Glucose	9.767
3	Sucrose	14.842

2.5.3 Patchouli alcohol analysis

2.5.3.1 Extraction method

Fifty grams of fresh *P. cablin* leaves was extracted with 400 mL of ethanol or hexane (99% purity) during 6 – 8 h in a conventional Soxhlet apparatus (500-mL boiler) (Figure 2.5). At the end of extraction, the liquid extract was filtered and evaporated to complete dryness in a vacuum at 35 °C using a rotary evaporator. Finally, the dried extract was stored at 4 °C for further studies. All experiments were performed in triplicate.

To identify the presence of patchouli alcohol in the *P. cablin* callus cultures, two types of cells were used; i.e. 1) fresh and friable callus cultures and 2) dark brown callus cultures. Both types of callus cultures were dried in the oven. About 300 mg of dried cells were powdered using mortar and pestle. The powder was then mixed with 6 ml of HPLC-grade methanol and homogenised for 5-10 minutes in the fume cupboard at room temperature. The mixture was sonicated for 30 minutes in the ultrasonicator and the slurry was centrifuged at 1800xg for 5 minutes to separate the cells and methanol. After removing the methanolic layer, the residue was extracted twice more with 3 ml methanol. All the combined methanol extracts were

evaporated to dryness at room temperature. The residue was dissolved in 2 ml of methylene chloride, with the addition of 2 ml distilled water. The mixture was first shaken for 10 seconds before centrifuged at 1700xg for 10 minutes. The methylene chloride (CH_2Cl_2) layer was collected and evaporated to dryness at room temperature (25 °C). Then, the residue was reconstituted in 2 ml of HPLC-grade methanol and filtered through a 0.2 μm membrane filter (Gelman Sciences) for GC analysis.

For the preparation of sample from *P. cablin* cell suspension cultures, the cell-free medium was extracted twice with equal volume of methylene chloride. After removal of the aqueous phase, the combined methylene chloride phase was evaporated at room temperature in the fume cupboard. The residue was then reconstituted in 1 ml of HPLC-grade methanol and filtered through a 0.2 μm membrane filter (Gelman Science) prior to GC analysis.

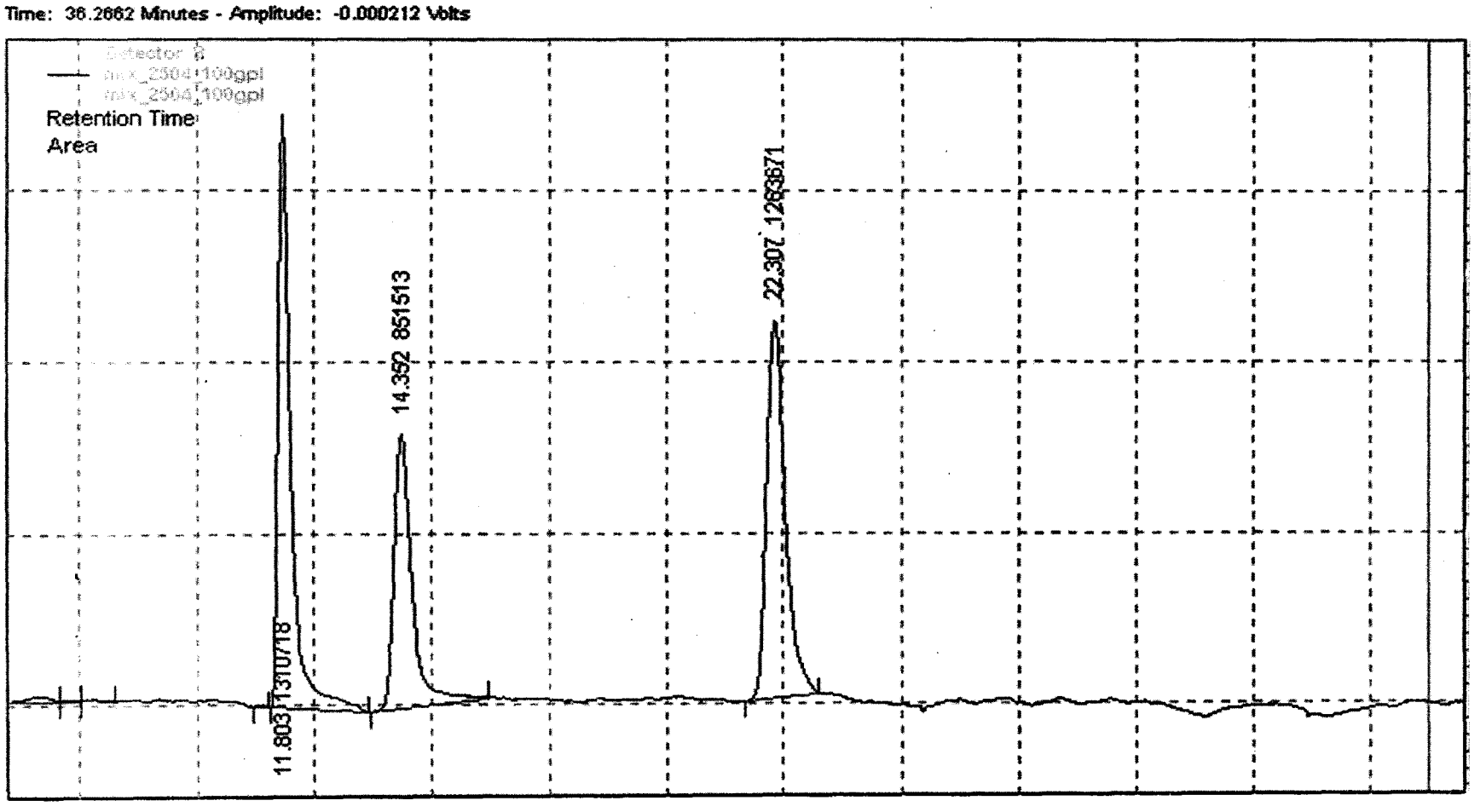

Figure 2.4: Chromatogram from HPLC for each sugar standard

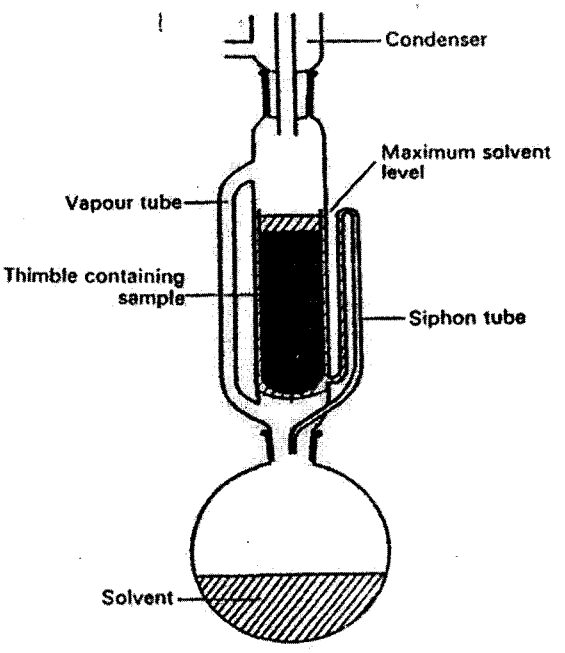

Figure 2.5: Soxhlet extraction apparatus

2.5.3.2 Identification of bioactive compounds using GC-MS analysis

The patchouli alcohol content in the leaf extract solution was analyzed by GC-MS model Clarus 600 Gas Chromatograph integrated with Clarus 600T Mass Spectrometer using column HP-5MS Agilent, 30 mm x 0.25 mm x 0.25µm. The analysis was done using helium as the carrier gas. The standard patchouli alcohol was purchased from Sigma-Aldrich and was identified by GC-MS to ensure the retention time. After that, the sample of leaf extract was analyzed and the compound was identified by comparing the retention time with the standard solution. The standard patchouli alcohol was detected at retention time of 17.734 and the peak of patchouli alcohol was presented in the chromatogram (Figure 2.6).

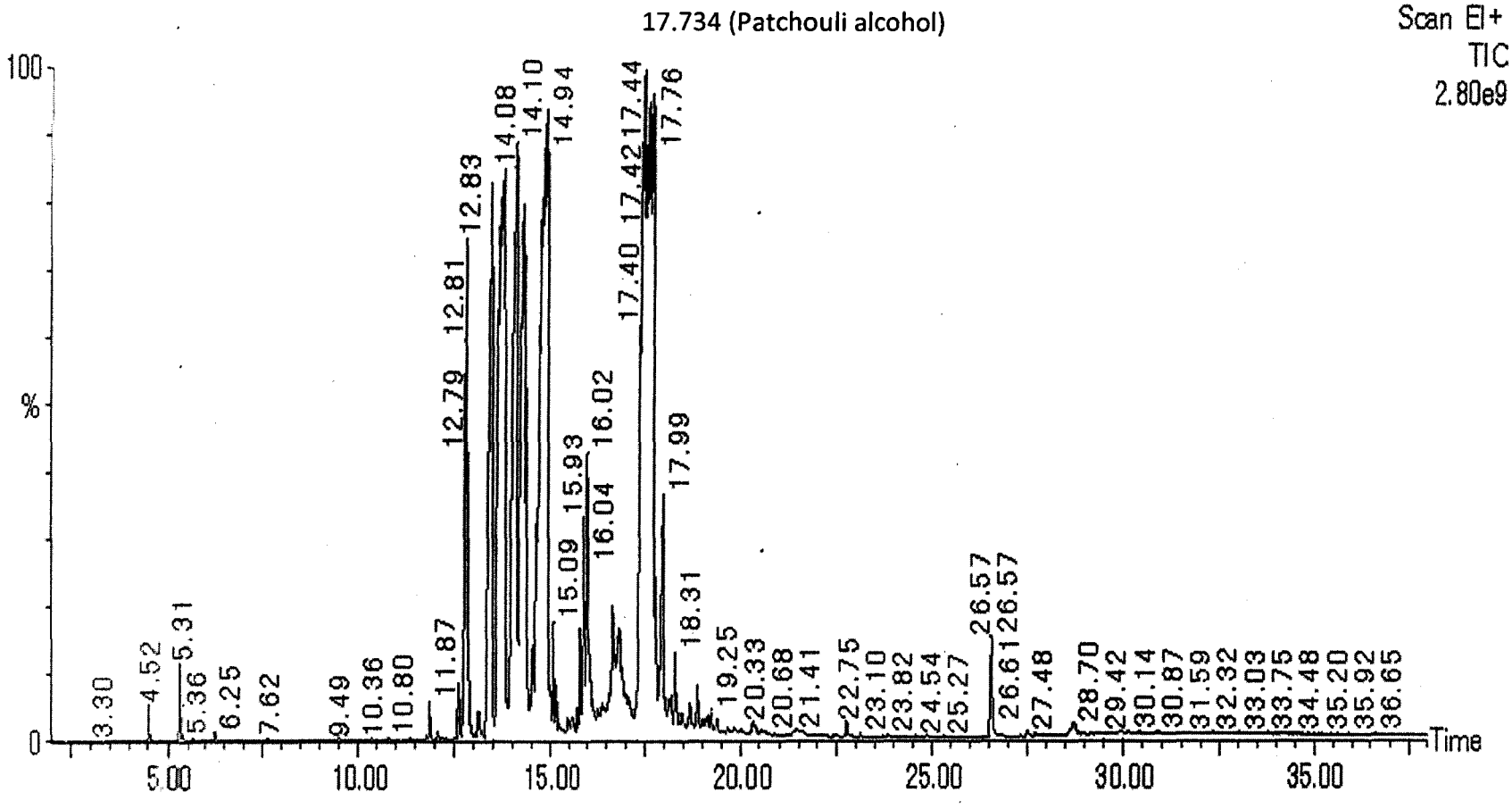

Figure 2.6: Chromatogram of the patchouli alcohol (standard) from GC-MS analysis

2.5.3.3 Quantification of patchouli alcohol using Gas Chromatography (GC)

Compounds were identified by comparing the Kovats GC retention indices of the peaks on the HP-5MS column with literature values, computer matching using the Masslynx database, and comparison of the fragmentation patterns of the mass spectra with those reported by Bunrathep *et al.* (2006). The Kovats index of patchouli alcohol was 1659 and an MS fragmentation pattern in the order of decreasing m/z value was (222 [M+], 205, 189, 161, 138, 125, 109, 95, 81, 67, 55, 41). Identification of compounds was also checked by using the standard patchouli alcohol. The concentration of patchouli alcohol was calculated with respect to the internal standard.

3.0 RESULTS AND DISCUSSION

3.1 Establishment of *P. cablin* callus cultures

3.1.1 Callus cultures initiation and maintenance

Callus cultures were induced from the *P. cablin* leaf explants. After 3 weeks of incubation, the cell cultures started to grow from the explants. The cells were transferred into the new solid medium for continuing the growth. The callus cultures were induced from explants with a small degree of formation. It takes about 3 - 4 weeks cultures. A few explants turned to brown and some of them did not induced any cells and it was similar with *C. asiatica* callus culture (Tan *et al.,* 2010). After two sub-cultured process, the cells were separated from the remained leaves explant and transferred into a new solid medium containing in jar bottle for a large surface area to avoid the cell died while still attached with the explants. In a first stage, the color of callus induction was light brown and compact. Figure 3.1 depicts the leaf explants of *P. cablin* and Figure 3.2 shows the callus induced from leaf explants.

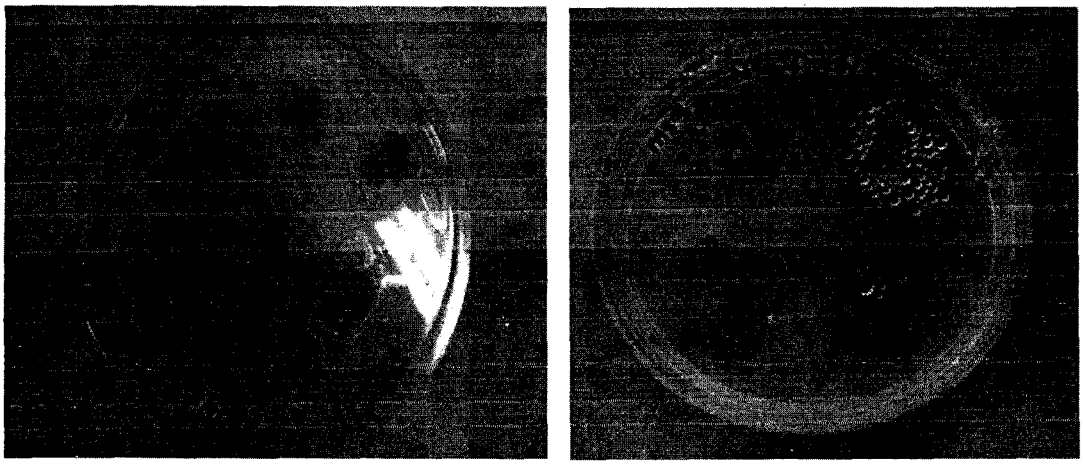

Figure 3.1: Leaf explants of *P. cablin*, cultured on the MS3 medium

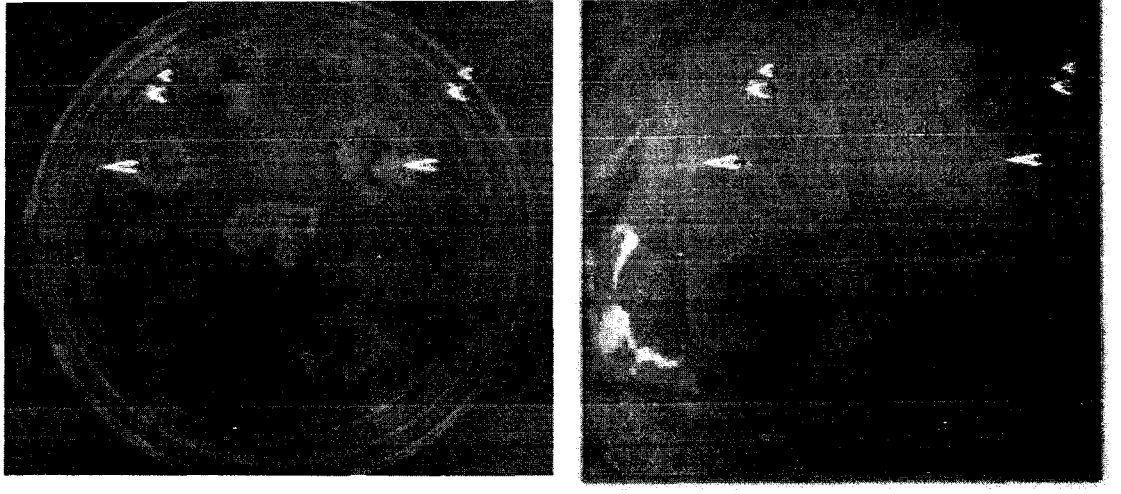

Figure 3.2: *P. cablin* callus cultures developed from the leaf explants and maintenance after several sub-culture

3.1.2 Effect of plant growth regulators and light condition on callus cultures growth

To maintain the best establishment of *P. cablin* callus cultures, the effects of plant growth regulator (PGR) and light condition have been studied. Cytokinins and auxins are the PGRs that play major role in plant growth and development. There are two types of auxin which are naturally occurring and synthetic. IAA and IBA are identified as the naturally occurring auxin, while NAA, 2, 4-D, and picloram are the synthetic auxin. Three types of PGR were used in this studies, which are NAA (0.25 - 1 mg/L), 2, 4-D (0.25 - 1 mg/L), and picloram (1 - 3 mg/L). From the previous study, naturally auxin is less effective than synthetic auxin. Several types of auxin and cytokinin give dissimilar effects of callus formation process (Tan *et al.*, 2010). Most of the callus cultures turned to brown after two weeks when cultured in the medium either with or without PGR. From Table 3.1 (a), 3.1 (b) and 3.1 (c), it was observed that callus cultures grown on MS1 media and maintained in the dark was the best condition and callus was formed in a very short period of time (21 days) compared to other medium combination. This was followed by callus cultures grown in medium MS10 under all three light conditions. A significant amount of callus cultures was also formed in MS3 medium in the dark, MS8 medium under continuous light condition and MS9 medium under photoperiod although longer time was needed (30 days). Among all the treatment, callus cultures formed in MS1 medium were healthy, watery

and friable with white and yellowish color. Nonetheless, in MS10 medium, the callus cultures produced were white, yellowish, watery and compact.

Table 3.1 (a): Influence of different concentration of PGRs on the establishment of *P. cablin* callus cultures under photoperiod (16 h light, 8 h dark) condition.

Media	Plant Growth Regulator (mg/L)			Callus color / morphology	Degree of callus formation	Time (day)
	BAP	NAA	2,4-D			
MS1	1			yellowish, greenish, watery	**	30
MS2	2	1		brownish, yellowish, watery	**	29
MS3	3			greenish, watery	***	30
MS4	1	0.25		yellowish, greenish, watery	**	30
MS5	1	0.75		yellowish, greenish, watery	**	28
MS6	1		0.25	dark brown, watery	**	30
MS7	1		0.75	dark brown, watery	**	30
MS8	2			brownish, yellowish, watery	**	30
MS9	3	1		greenish, watery	***	29
MS10		1		yellowish, white, greenish, watery	***	25
MS11	1	1		yellowish, greenish, watery	**	30
MS12	1		1	dark brown, watery	*	28
MS13	1		0.5	whitish, friable	***	25
MS14	1	0.5		brownish, friable	***	28
MS15			1	brownish, watery	*	30
MS16	2		1	brownish, watery	*	30
MS17	3		1	brownish, watery	*	30

*callus formed <1 cm; **callus formed between 1.5-2 cm; ***callus formed more than 2.5 cm

Table 3.1 (b): Influence of different concentration of PGRs on the establishment of *P. cablin* callus cultures under continuous light condition.

Media	Plant Growth Regulator (mg/L)			Callus color / morphology	Degree of callus formation	Time (day)
	P	NAA	2,4-D			
MS1	1			yellowish, greenish, watery	**	30
MS2	2	1		yellowish, watery	**	30
MS3	3			greenish, watery	***	30
MS4	1	0.25		dark brown, watery	*	30
MS5	1	0.75		yellowish, greenish, watery	**	30
MS6	1		0.25	brownish, watery	*	30
MS7	1		0.75	brownish, watery	**	30
MS8	2			greenish, watery	***	30
MS9	3	1		yellowish, watery	**	30
MS10		1		dark brown, watery	*	30
MS11	1	1		yellowish, greenish, watery	**	30
MS10		1		yellowish, white, greenish, watery	***	25
MS11	1	1		yellowish, greenish, watery	**	30
MS12	1		1	dark brown, watery	*	30
MS13	1	-	0.5	brownish, watery	*	30
MS14	1	0.5	-	brownish ,watery	***	30
MS15	-	-	1	brownish ,watery	*	30
MS16	2	-	1	brownish ,watery	*	30
MS17	3	-	1	brownish ,watery	*	30

*callus formed <1 cm; **callus formed between 1.5-2cm; ***callus formed more than 2.5 cm

induction in Carob (*Ceratonia siliqua*) in which 2, 4-D supported the formation of small callus which turned to brown and failed to develop. However, the low concentration of 2.4-D does not affect callus development (Carimi *et al.,* 1997). Based on the observation, the callus maintained in the dark condition grew very well than the callus maintained under photoperiod or exposed to continuous light. The callus also became darker possibly because of the production and oxidation of phenolic compounds from the explants (Soomro and Memon, 2007). Figure 3.3 shows the texture of the callus in the dark, photoperiod and continuous light conditions, respectively.

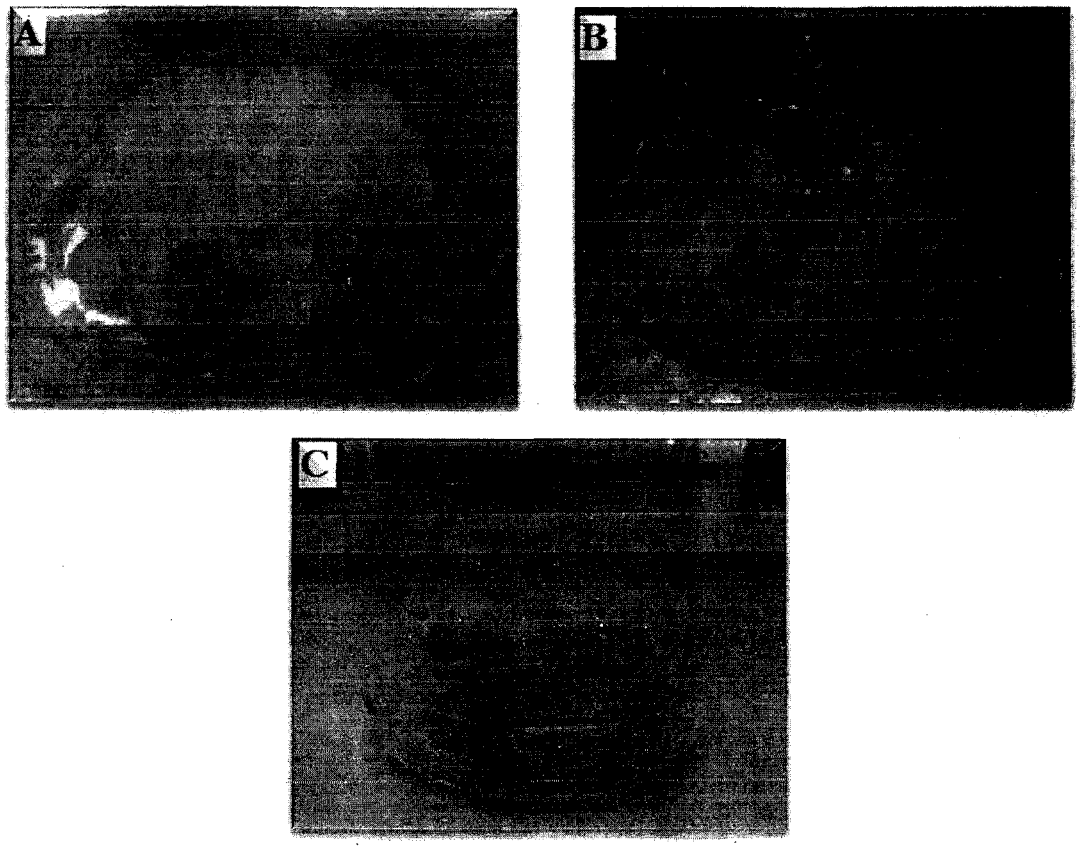

Figure 3.3: Callus cultures growth in the medium containing 1 mg/L P and maintained in different conditions: (A) dark, (B) photoperiod and (C) continuous light

3.2 Establishment of *P. cablin* cell suspension cultures

3.2.1 Growth kinetics studies (batch experiment)

3.2.1.1 Measurement of cell growth and carbohydrate consumption

The cell suspension cultures of *P. cablin* were maintained in the dark condition to study the growth kinetics. Figure 3.4 presented the carbohydrate consumption during the growth of *P. cablin* cell suspension cultures. Based on the growth profile, it shows that the content of sucrose is decreased with the increasing incubation time of cell suspension cultures and completely utilized after 14 days. The sucrose was hydrolyzed to fructose and glucose starting from 0 day. Sucrose has been widely used as a sole carbohydrate source of plant culture medium (Mello *et al.*, 2001). When the medium was sterilized at 121 °C, one–sixth of the sucrose is also hydrolyzed into glucose and fructose. Sucrose was quickly absorbed and/or totally hydrolyzed in the cell suspension cultures (Son *et al.*, 2000). These results indicate that sucrose for both cell clumps culture medium and protoplast culture medium might be due to the partial breakdown of sucrose into glucose and fructose by autoclaving (Godo, Matsui *et al.* 1996). The concentrations of fructose and glucose were slightly increased at day 1 due to hydrolysis of sucrose. The cells consumed minimum fructose on day 2 but glucose was preferred to support the growth of the cells as can be seen from the depletion of glucose by day 6. Subsequently, fructose was accumulated in the medium, with the maximum concentration of 21.94 g/L was detected on day 6. The decrement of fructose in the medium was detected after both sucrose and glucose were completely consumed by the cells after day 6. The complete depletion of sugars in the medium is in accordance to the reduction of fresh and dry weight after day 14 of incubation (Figure 3.5).

Table 3.1 (c): Influence of different concentration of PGRs on the establishment of *P. cablin* callus cultures in the dark condition

Media	Plant Growth Regulator (mg/L)			Callus color / morphology	Degree of callus formation	Time (day)
	BAP	NAA	2, 4-D			
MS1	1	-	-	white, yellowish, watery	***	21
MS2	2	1	-	greenish, watery	**	30
MS3	3	-	-	white, yellowish, watery	***	21
MS4	1	0.25	-	yellowish, greenish, watery	**	27
MS5	1	0.75	-	yellowish, greenish, watery	**	27
MS6	1	-	0.25	brownish, watery	*	27
MS7	1	-	0.75	dark brown	*	27
MS8	2	-	-	greenish, watery	**	30
MS9	3	1	-	greenish, watery	**	30
MS10	-	1	-	white, yellowish, watery, friable	***	25
MS11	1	1	-	yellowish, greenish, watery	**	27
MS12	1	-	1	brownish, watery	*	27
MS13	1	-	0.5	brownish, watery	***	30
MS14	1	0.5	-	yellowish, watery	***	28
MS15	-	-	1	brownish, watery	*	30
MS16	2	-	1	brownish, watery	*	30
MS17	3	-	1	brownish, watery	*	30

*callus formed <1 cm; **callus formed between 1.5-2cm; ***callus formed more than 2.5 cm

The different concentrations of PGR also exhibit a considerable effect on callus formation process. However, the medium supplemented with different concentration of 2, 4-D (MS6, MS7, MS12) in all three light conditions showed the less callus cultures formation and the color of the callus turned to dark brown after 25 days. This observation is in line with the callus

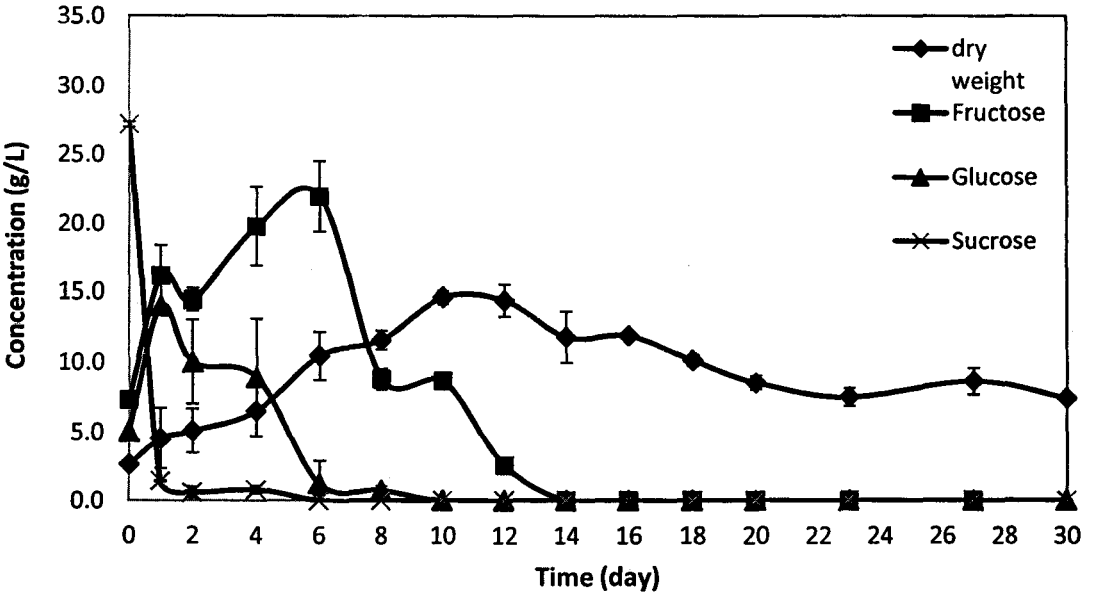

Figure 3.4: Relationship between cell growth and sugar consumption in *P. cablin* cell suspension cultures in batch experiment.

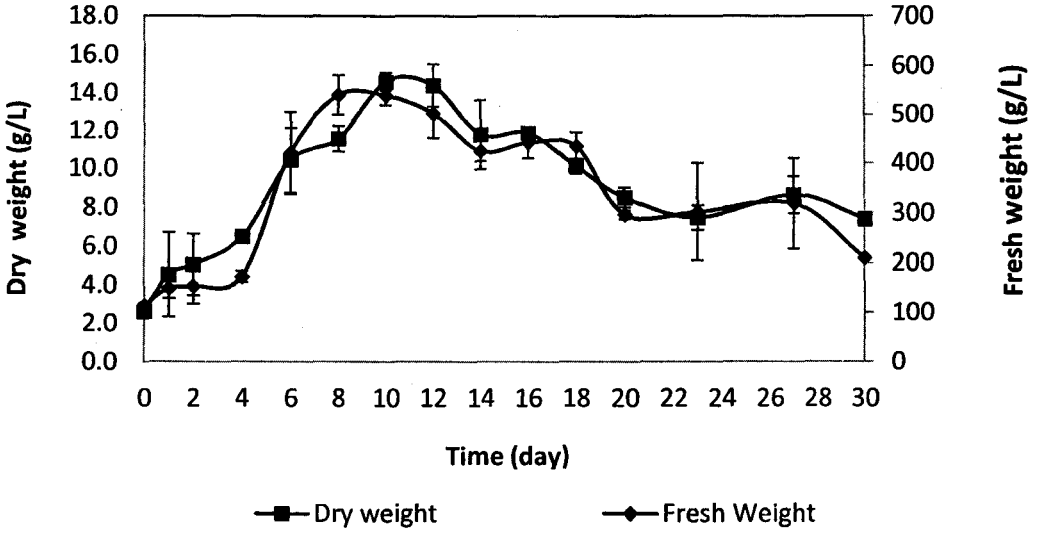

Figure 3.5: The fresh and dry weight measurements for *P. cablin* cell suspension cultures

3.2.1.2 Measurement of medium pH

Figure 3.6 illustrates the pH profile measured in the medium. The pH was stabilized from the beginning until day 4 of incubation started to increase to 6.5, parallel to the increment of cell dry weight. The similar trend was also observed for Parsley and soybean cell suspension cultures. The changes of pH in the medium were probably due to the uptake and utilisation of nitrogen sources (ammonium and nitrate) available in the medium by the cells. Later, the medium pH has slightly increased and maintained while the cell cultures enter the stationary and death phase after 16 days.

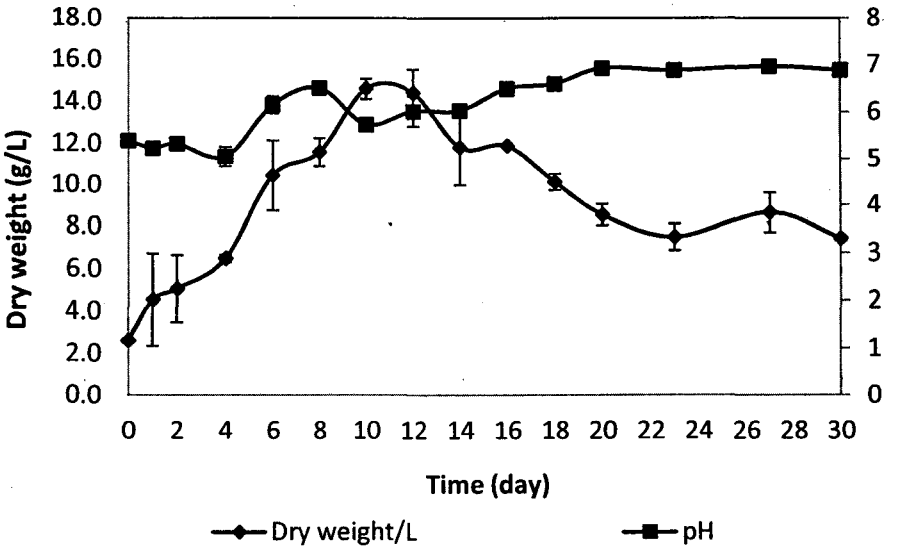

Figure 3.6: Time course of pH measurement for *P. cablin* suspension cultures

3.2.2 Effect of plant growth regulator and light on *P. cablin* cell suspension cultures

The effects of PGRs and light have been studied on the growth of *P. cablin* cell suspension cultures. Generally, the medium composition and physical factor such as light has significant influence on the development of cell cultures (Behbahani *et al.*, 2011). In this section, the cell suspension cultures were grown in three different conditions, i.e. under continuous light, photoperiod and in the dark. These conditions were chosen based on the observation from the result of callus cultures establishment. The callus cultures grown under continuous light was proved to be unfavourable to support good growth. From the growth profile of cell suspension cultures, it was observed that all the cell lines displayed a sigmoidal curve. When the cell suspension cultures were grown in the dark condition (as shown in Figure 3.7), cell suspension cultures from flask MD1 (12.03 g FW; 0.5 g DW) exhibited good growth rate and the cells is whitish in color. This was followed by MD2 (11.06 g FW; 0.46g DW) but the cell showed a brown color, while MD3 (10.71 g FW; 0.41g DW) displayed the whitish color of cells. For other PGRs combination, the cells exhibited slow growth and produced a small amount of biomass. The growth trend of *P. cablin* cell suspension cultures in all medium showed that the cells grew slowly at the first 3 days (lag phase) but increased exponentially from day 4 to day 12 (exponential phase) and reached stationary phase after day 15.

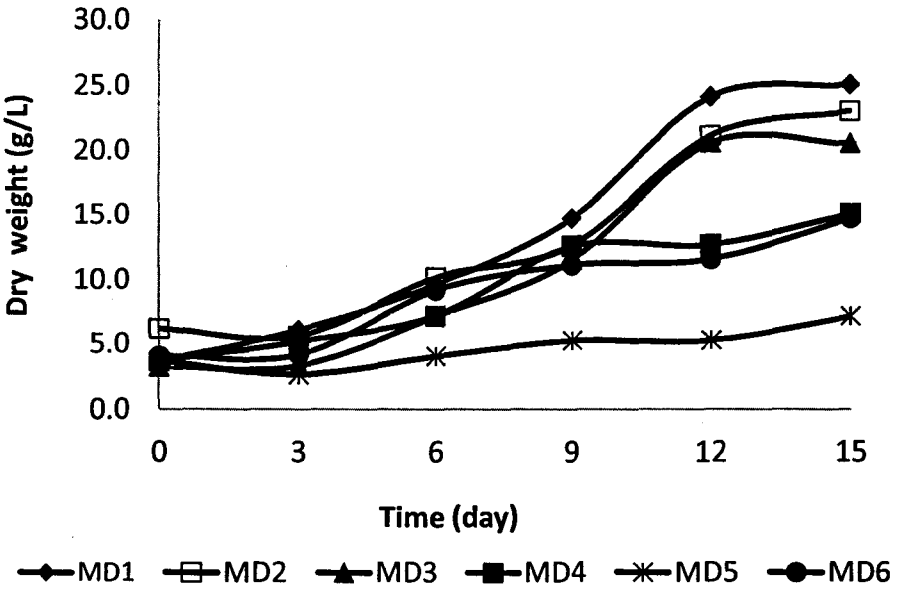

Figure 3.7: Growth profile of *P. cablin* cell suspension cultures grown for 15 days in the dark condition. The legends were defined in Table 2.2.

Meanwhile, the cell suspension cultures that were maintained in photoperiod condition also showed a good growth profile, except for MP5 (Figure 3.8). Similar results were observed on growth and development of *Rollinia mucosa* cell suspension cultures, in which picloram was used as the plant growth regulator (Figueiredo *et al.*, 2000). As depicted in Figure 3.8, the exponential phase of MP1 is faster while the lag phase is shortest than others. However, a significant amount of cells was also obtained from MP2, MP3 and MP6 as indicated by the dry weight measurement, although the lag phase is the longest. This is possibly because the cells were adapting to the new environment during the lag phase and started to consume the nutrients as they have adapted to the environment and eventually grew exponentially. It was observed that the cells did not grow well in the medium containing the combination of 2, 4-D (1 mg/L) and BAP (1 mg/L), maintained under both light conditions (i.e. MD5 and MP5). For continuous light condition, MC2 showed the highest biomass and the cells did not grow well in MC6 containing the combination of 2, 4-D and BAP (Figure 3.9). Nonetheless, when the cells were grown on these individual PGRs (information as given in Table 4.2), they exhibited good

growth rate. This shows an inhibitory effect on the cell growth when these PGRs were supplied at equal amount, but promoted good cell growth when the concentration of BAP (cytokinin) was reduced by half, as obtained in flasks MD6 and MP6, respectively. Based on the summarized results as tabulated in Table 3.2, it was apparent that the supplementation of 1 mg/L picloram could promote good growth when the cell suspension cultures were grown in the dark condition (MD1) as compared to photoperiod (MP1) and continuous light (MC1).

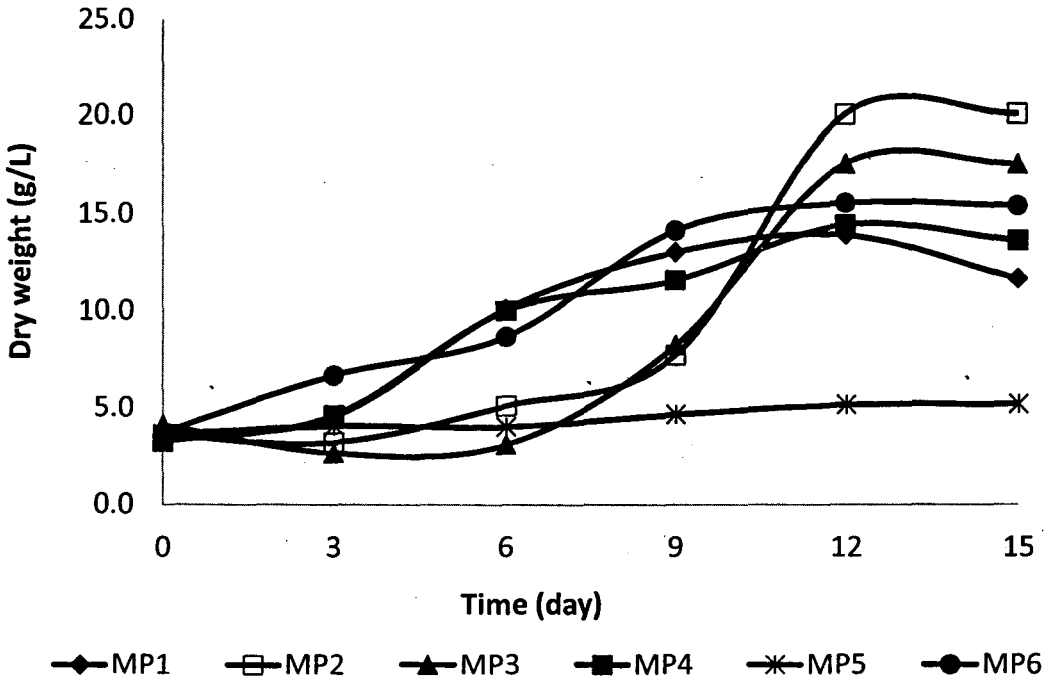

Figure 3.8: Growth profile of *P. cablin* cell suspension cultures grown under photoperiod condition for 15 days. The legends were defined in Table 2.2.

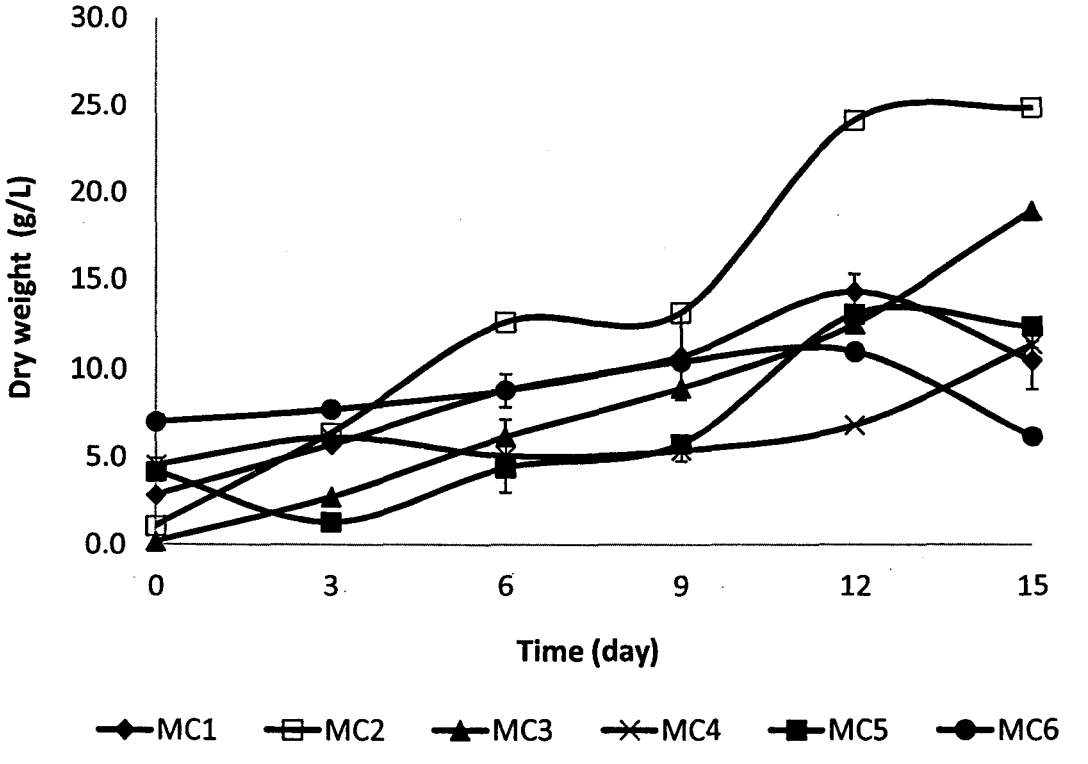

Figure 3.9: Growth profile of *P. cablin* cell suspension cultures grown under continuous light for 15 days. The legends were defined in Table 2.2.

Table 3.2: Effects of different combination and concentration of PGR and light condition on the growth of *P. cablin* cell suspension cultures.

Light Condition	Medium	Colour of suspension cultures	Fresh weight (g/L)	Dry weight (g/L)
Dark	**MD1**	**Whitish**	**601.51**	**25.07**
	MD2	Brownish	553.10	23.06
	MD3	Whitish	535.94	20.55
	MD4	Brownish	444.28	15.07
	MD5	Light brown	290.09	7.12
	MD6	Yellowish	434.71	14.68
Photoperiod	MP1	Brownish	431.64	11.65
	MP2	Brownish	492.59	20.09
	MP3	Whitish	535.24	17.55
	MP4	Brownish	522.62	13.63
	MP5	Brownish	184.71	5.17
	MP6	Light green	552.39	15.43
Continuous light	MC1	Whitish	493.56	10.51
	MC2	Brownish	509.80	24.88
	MC3	Brownish	407.95	18.94
	MC4	Brownish	316.30	11.39
	MC5	Brownish	212.58	12.40
	MC6	Brownish	203.43	6.21

3.2.3 Influence of carbohydrates on the growth of *P. cablin* cell suspension cultures

Three different carbohydrates, i.e. sucrose, fructose and glucose, with different concentration and combination were assessed to investigate their effects on the growth of *P. cablin* cell suspension cultures. Table 2.3 shown previously has summarized the nine set of carbohydrate combinations and concentration used in this study. The influence of carbohydrate was examined based on the fresh and dry weight analysis.

Based on the Figure 3.10 below, it shows the trend of sigmoidal growth curve. The cell cultures grew well in the medium supplemented with glucose, reaching the highest cell dry weight of 13.8 g/L, followed with sucrose (8.9 g/L) and fructose (1.9 g/L) on day 15. Besides, different plants required different carbon source due to the different enzymatic metabolism (Ong *et. al.,* 2008). Sugars play multiple roles in all aspects of plant life (Yu, 1999). The lag phase of cells in the medium supplemented in glucose is the shortest compared to fructose and sucrose. On the other hand, Figure 3.11, 3.12and 3.13 described the relationship between sugar uptake and cell growth (by dry weight measurement), respectively. The uptake rate was decreased when the time increased. However, fructose uptake at day 0 was beyond than expected.

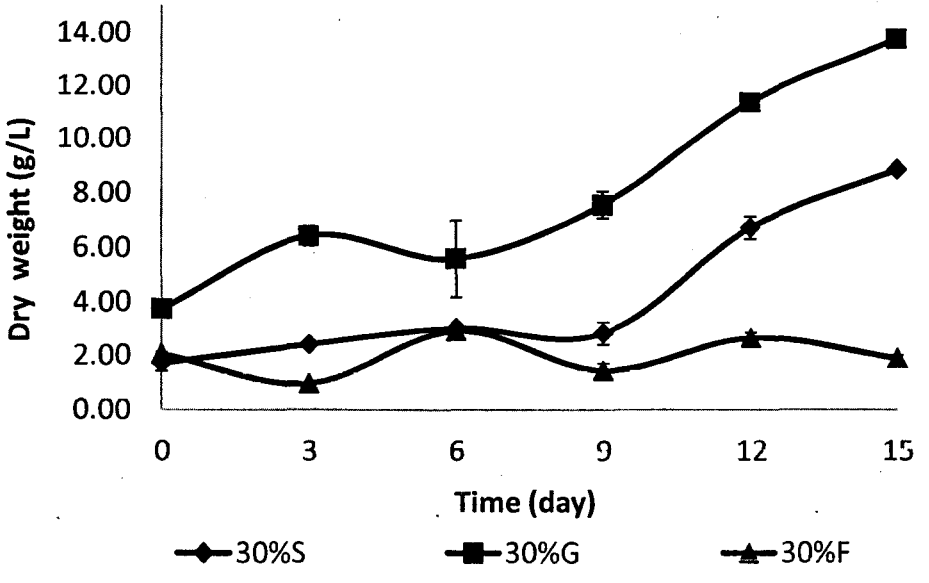

Figure 3.10: Effect of different carbohydrates on the growth of *P. cablin* cell suspension cultures.

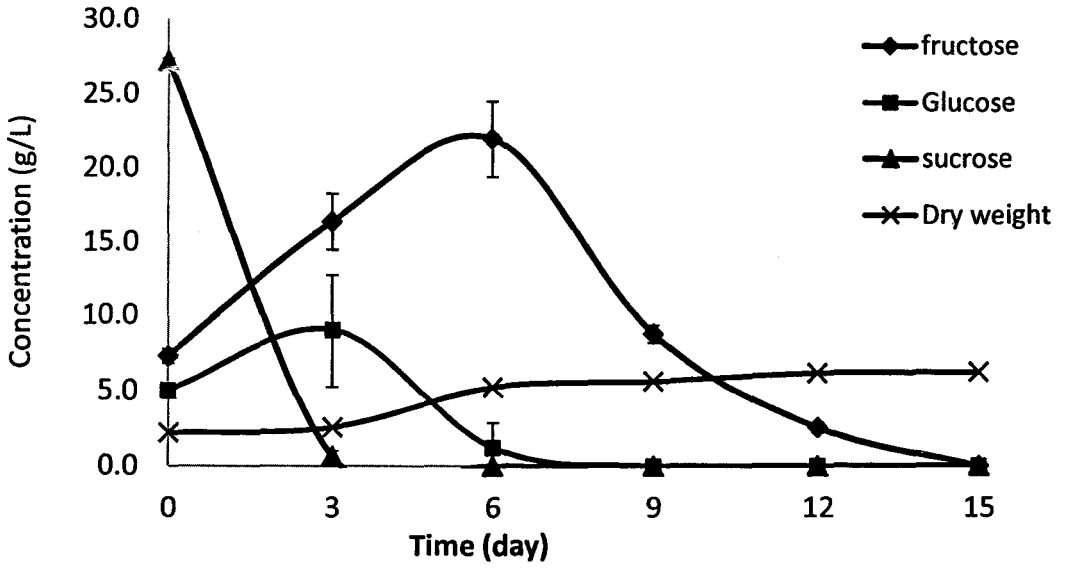

Figure 3.11: Relationship between cell growth and sucrose consumption in *P. cablin* cell suspension cultures (Set A)

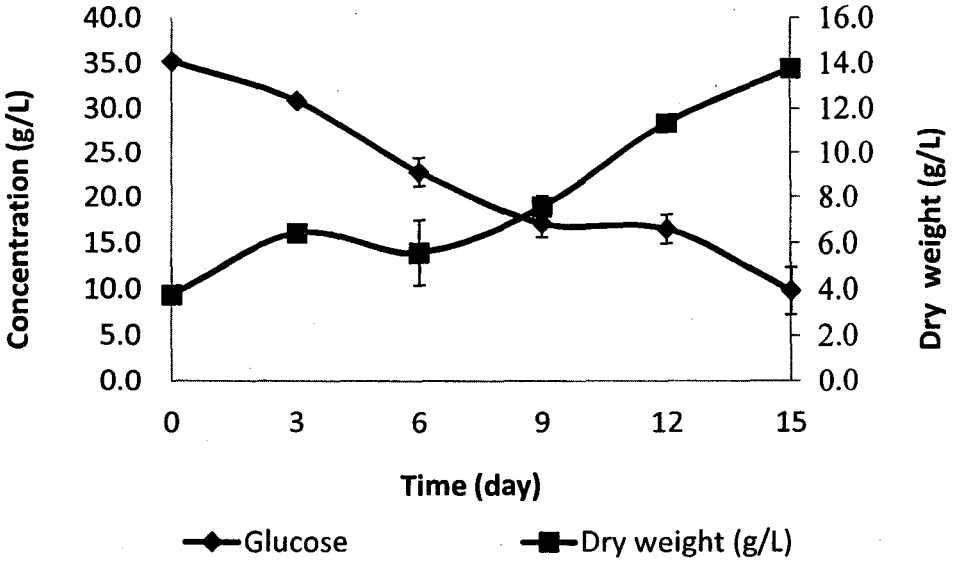

Figure 3.12: Relationship between cell growth and glucose consumption in *P. cablin* cell suspension cultures (Set B)

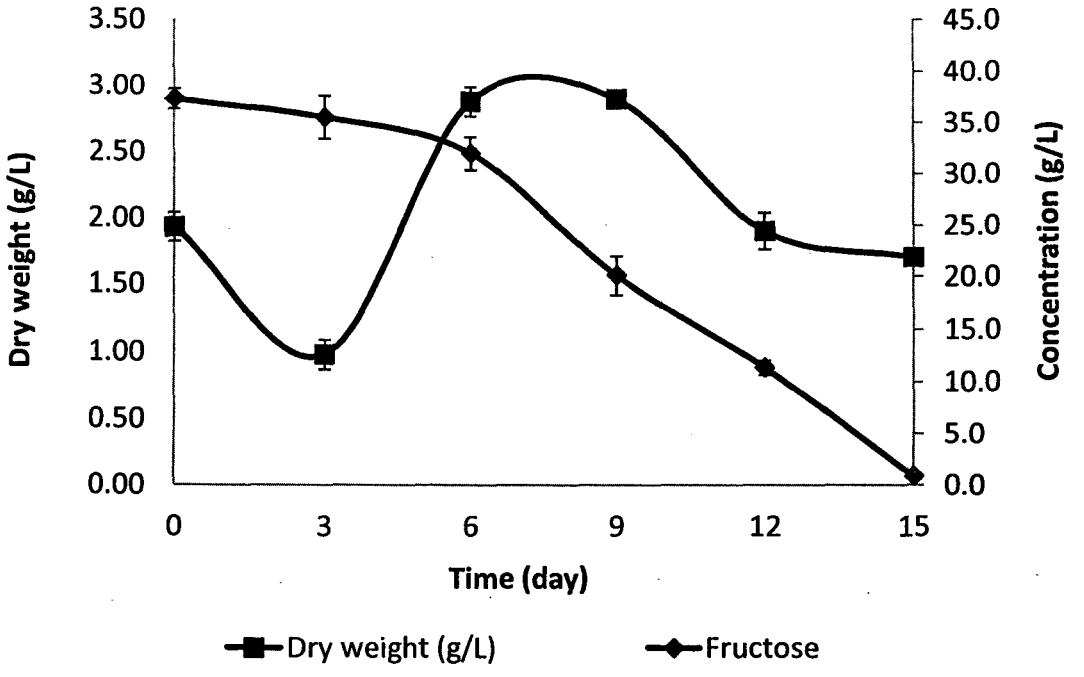

Figure 3.13: Relationship between cell growth and fructose consumption in *P. cablin* cell suspension cultures (Set C)

The cell suspension cultures supplemented with equal amount of glucose and fructose (G2F2) (Set E) achieved the highest biomass compared to Set D (G2F4) and F (G4F2). At day 0 to day 9, cells grew well in Set E, but not for the other two combinations. The ratio of glucose and fructose supplied in the medium has influenced the growth of the cell. When the cells were grown in the medium containing high glucose concentration than fructose, they exhibited slightly better growth profile as shown in Figure 3.14. The effect of glucose on cell growth of in association with the presence of fructose has facilitated cell separation on proliferation in suspension culture. The reason might be the lower glucose concentration had less effect on growth speed (Duong *et. al.*, 2006).

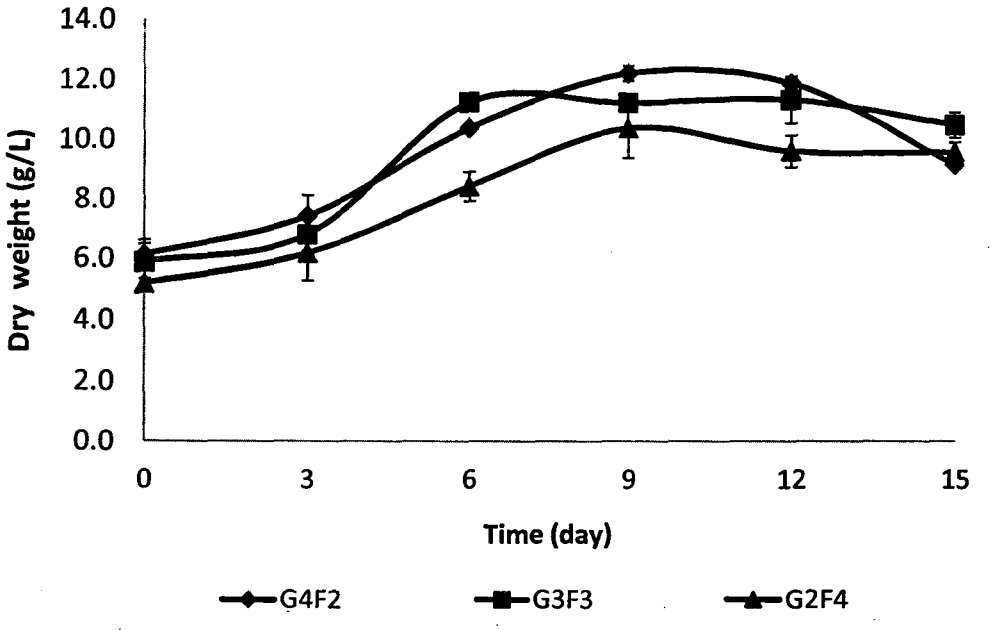

Figure 3.14: Effect of different combination and concentration of glucose and fructose on the growth of *P. cablin* cell suspension cultures

The effect of different sucrose concentration on the growth of *P. cablin* cell suspension cultures was studied by varying the preliminary medium concentration which are labeled as Set G, H, I and A (20, 60, 80, 30 g/L) (as defined in Table 2.3). Figure 3.15 shows the dry weight of each set for 15 days of cell cultivation. Growth profile of the cells grown in Set A illustrates the significant sigmoidal curve compared to other sets. Set G and A achieved the exponential phase at day 6 while the cell growth in Set H and I accelerated rapidly starting from 0 day. The maximum cell growth was obtained in Set H (14.59 g/L) whereas set I produced the minimum cell dry weight of 2.26 g/L. The similar trend was observed with *Coleus blumei* and *Perilla frutescens* cell cultures when 60 g/L of initial sucrose concentration was supplied in the growth medium (Wang *et al.*, 1999). From the result, it can be concluded that the higher initial sucrose concentration which is 80 g/L had suppressed the cell growth because of higher the osmotic pressure. The trend obtained in this study is in accordance to *Vitis vinifera* and *Holarrhena anti dysenterica* cell cultures. However, for *C. roseus* cell cultures, it was found that the active biomass cultivated in medium containing different concentration of glucose was approximately

unaffected. This might be due to phosphate and nitrate inside the medium as an inhibitor to limit the nutrient uptake by the cells.

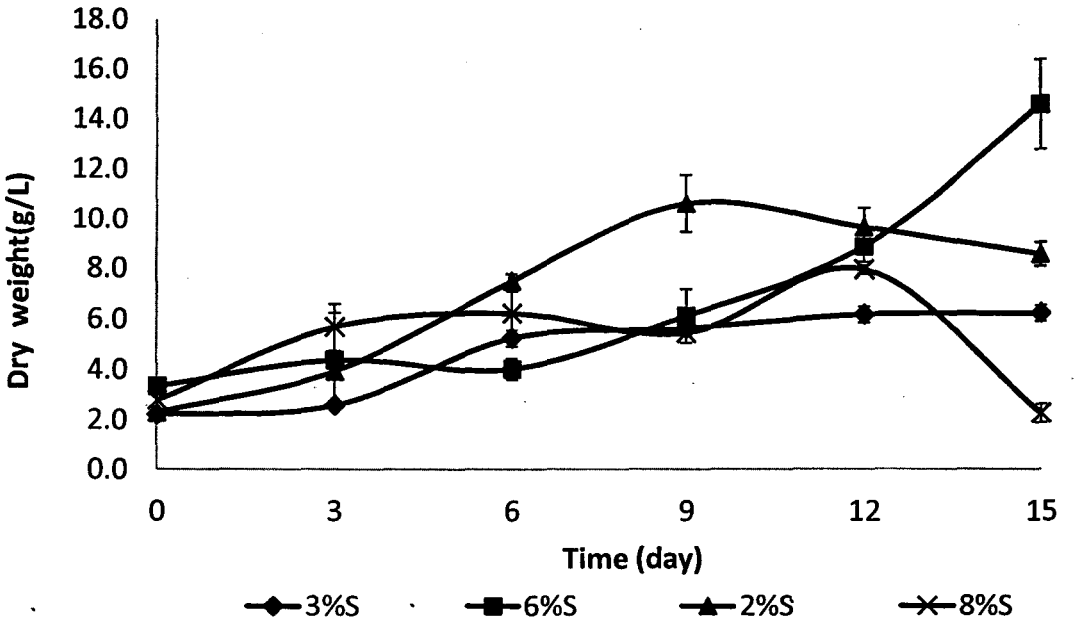

Figure 3.15: Effect of different sucrose concentrations on the growth of *P. cablin* cell suspension cultures

The trend of the sugars consumption was also similar in *Panax notoginseng* cultures (Zhang *et al.*, 1996). From Figure 3.12 and 3.16, glucose and fructose was produced in the medium from the hydrolysis process of the enzyme sucrose invertase in the cells. In all experiments, it was observed that the glucose was preferentially taken up by the cells and it was consumed at higher rate than fructose in Set A and I, respectively. However, in the experiment where sucrose was supplied excessively, i.e.: (Set H (6%) and Set I (8%)), the cells consumed fructose and glucose almost at equivalent rate. In Set H, both glucose and fructose in the medium was utilized by the cells to support the growth until day 9. Then, the cells consumed glucose only until day 15, leaving about 40 g/L of fructose in the medium. In Set I, where the excessive amount of sugar was supplied in the medium, the sucrose hydrolysis has

occurred rapidly at the beginning of cell culture growth. The rapid sucrose hydrolysis was supported with the accumulation of high concentration of glucose and fructose in the medium. Similarly, the cells consumed both glucose and fructose at almost equivalent rate, but the consumption is very slow as indicated by the increase concentration of these sugars in the medium. The dry weight profile in Figure 3.15 showed that the cells did not grow well although excessive carbon source was available in the medium. The reason being is that the high initial concentration of sucrose has created a higher osmotic pressure in the cell condition and a growth inhibitory effect by fructose which has affected the growth of the cells. The same trend had been observed in *Nicotiana tabacum* and *Cinchona succirubrum* cell cultures when they were grown on high fructose concentration in the medium (Nigra *et. al*, 1990).

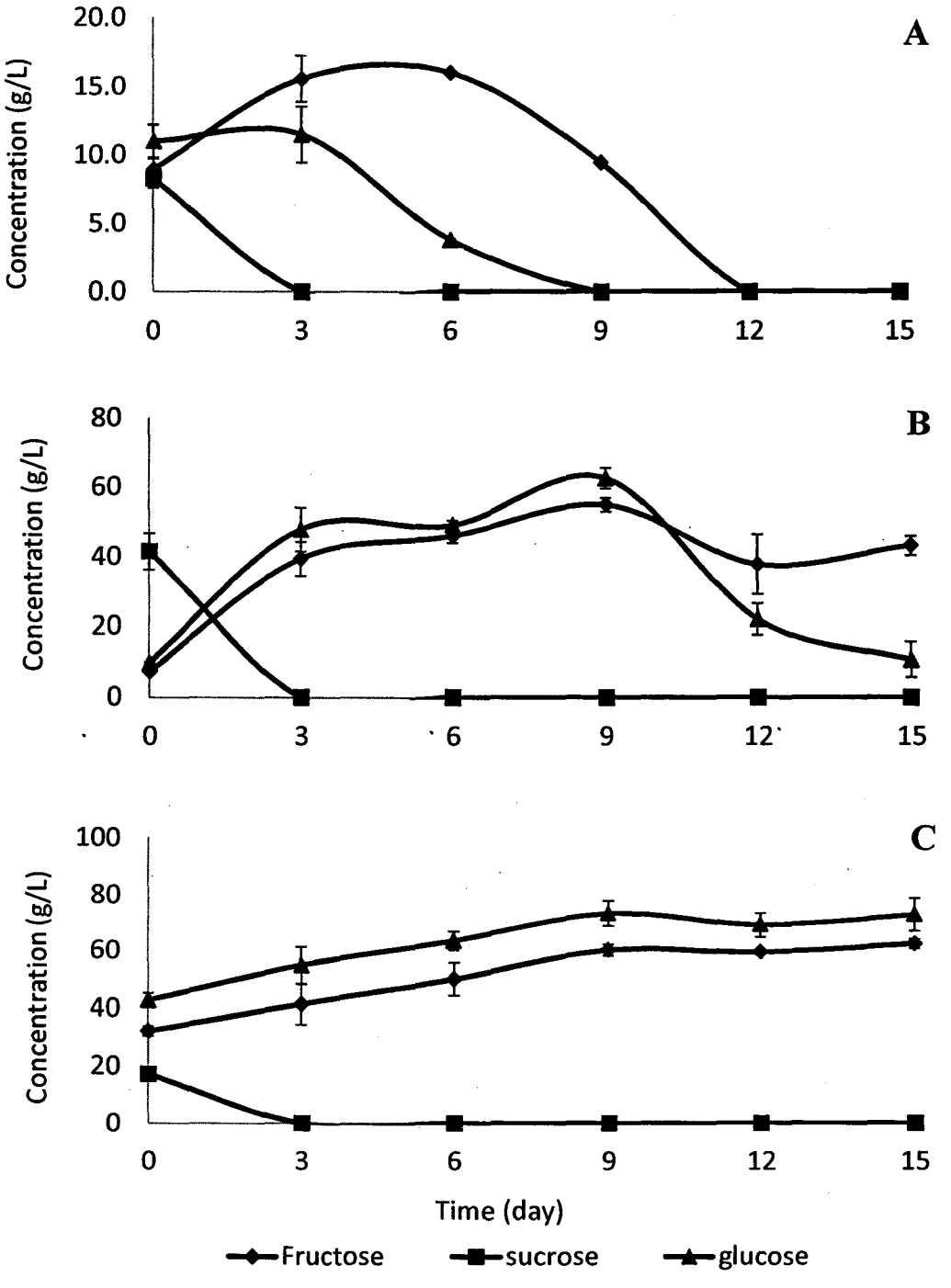

Figure 3.16: Sugar consumption profile for the growth of *P. cablin* cell suspension cultures; A: 2% sucrose (Set G); B: 6% sucrose (Set H); C: 8% sucrose (Set I)

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

The different inoculum size was investigated in growth of cell suspension culture of *P. cablin*. Three of inoculum sizes (w/v) which is 10% (2 g/20 ml), 15% (3g/20ml) and 20% (4g/20ml) were selected and the growth of cells was studied for 15 days cultivation. As shown in Figure 3.17, the highest biomass production was observed in 10% inoculum size treatment with 24.95 g/L. For both 20% and 15% inoculums size treatment, 19.48 g/L and 16.95 g/L of cells (dry weight) were measured, respectively. Treatment of 10% inoculum size showed sigmoidal growth curve compared to others. The cell growth achieved 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 % and 20% inoculums size treatment was longer until day 6 and entered the exponential phase later, then reached the stationary phase on day 12. It was observed that the higher the inoculum size, the lower the growth rate and biomass production. According to Ong *et al.* (2008), a minimum inoculum size was required in cell suspension cultures of plant cell. Guo and Zhang (2005) 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. This indicates that plant cells required a critical minimum inoculum density for good cell proliferation as well as for secondary metabolite production.

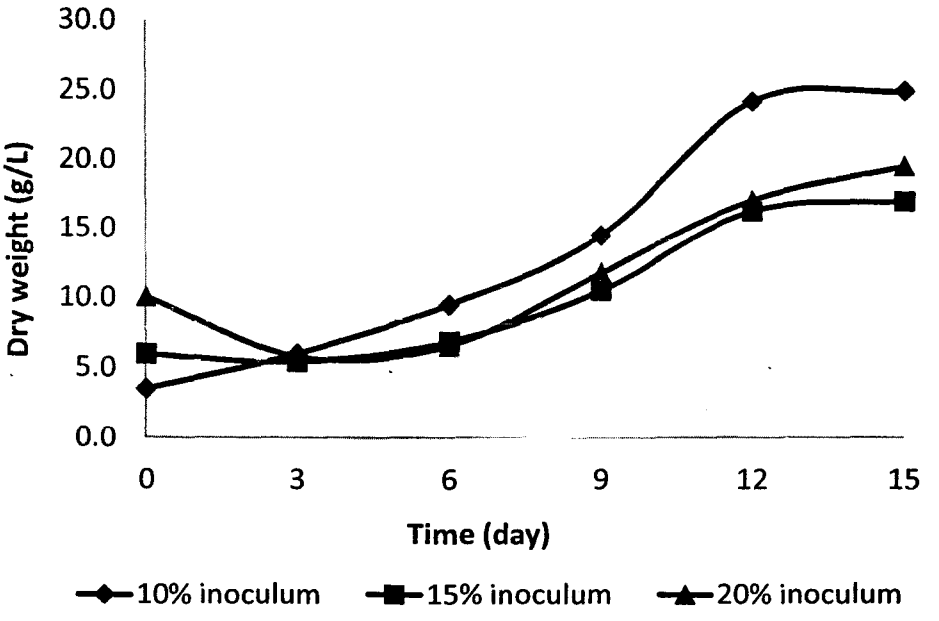

Figure 3.17: Effect of inoculum sizes on the growth of *P. cablin* cell suspension cultures

3.3 Patchouli alcohol production

Patchouli alcohol production was identified from the three sources, i.e.: leaf extraction, callus and cell suspension cultures of *P. cablin*. The GC-MS analysis showed the presence of eleven significant compounds and with the major compounds are patchouli alcohol (32.68 %) and Azulene (18.46%). The identified compounds are similar to the results obtained by Bunrathep *et. al.*, (2006). Figure 3.18 presents the chromatogram of compounds in the *P. cablin* leaf extract, while the name of the bioactive compounds is summarized in Table 3.3. However, patchouli alcohol could not be detected from the extracts of callus and cell suspension cultures, which is contrast to the results reported by Bunrathep *et. al.*, (2006). This could be because of several reasons: 1) the cells were grown and maintained in different medium that could not promote the production of patchouli alcohol (as the secondary metabolite), 2) the solvents used for extraction were not suitable to extract out the required compound from dried callus and cells from the suspension cultures, 3) the concentration of patchouli alcohol in these cultures was too low that could be detected by the GC-MS. Hence, further improvement either

on the growth conditions of the cells (use different medium or enhancement strategies such as precursor addition or elicitation) or on the analysis can be done in order to promote the production of patchouli alcohol in the plant cell culture systems.

Table 3.3: Chemical constituents obtained from *P. cablin* leaf extract

No.	Compound	Retention Time	Area %
1.	Azulene, 1,2,3,4,5,6,7,8-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1S-(1à,4à,7à)]-	13.73	10.38
2.	Azulene, 1,2,3,3a,4,5,6,7-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1R-(1à,3aá,4à,7á)]-	14.03	4.31
3.	à-Patchoulene	14.21	4.08
4.	Azulene, 1,2,3,5,6,7,8,8a-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1S-(1à,7à,8aá)]-	14.86	18.46
5.	Globulol	16.80	1.1
6.	Ledol	17.31	2.49
7.	**Patchouli alcohol**	**17.63**	**32.68**
8.	4-Methoxy-2,2,5-trimethylcyclopent-4-ene-1,3-dione	18.01	1.11
9.	1,4-Methanoazulen-9-ol, decahydro-1,5,5,8a-tetramethyl-, [1R-(1à,3aá,4à,8aá,9S*)]-	18.38	0.51
10.	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	19.42	0.50
11.	Phytol	22.88	1.22

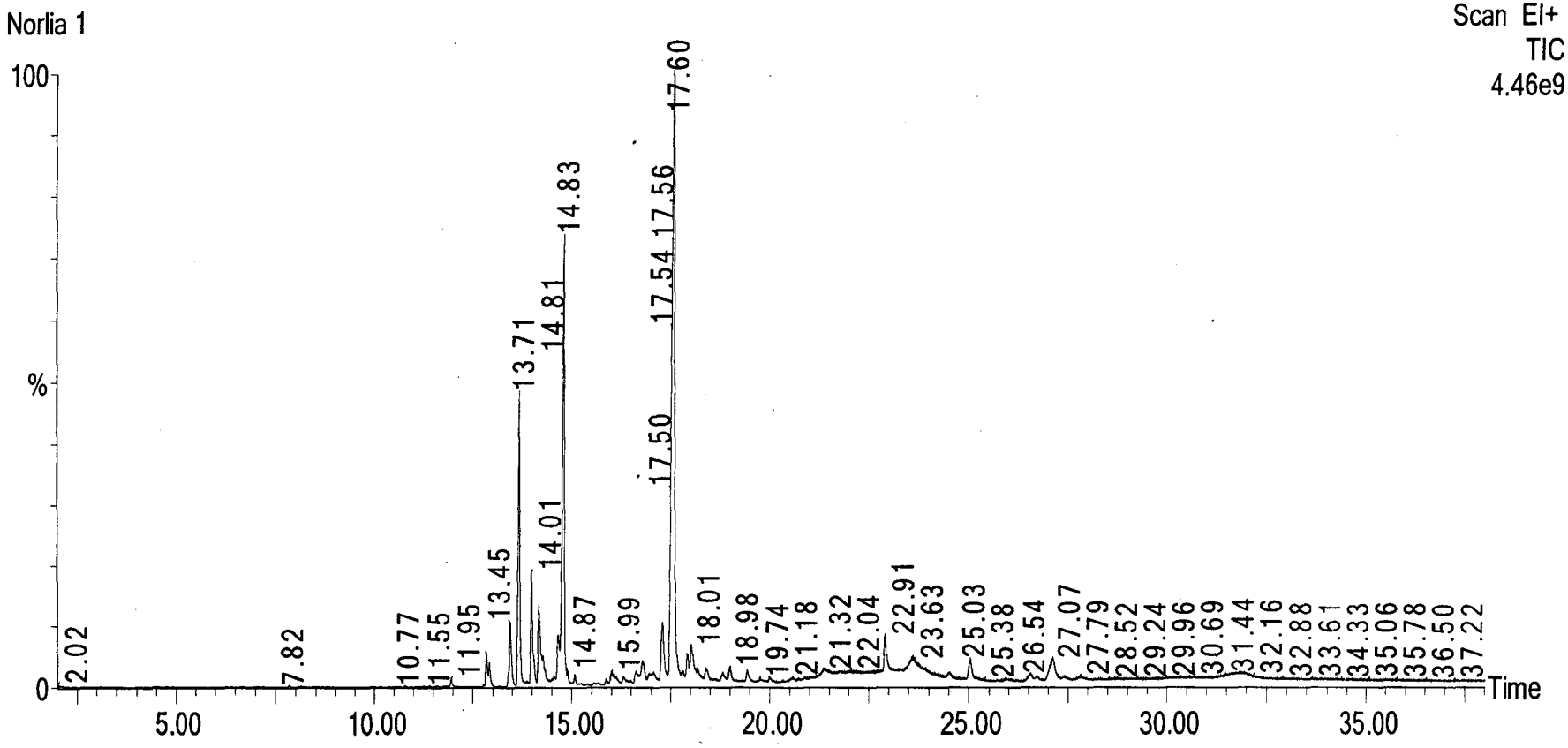

Figure 3.18: Chromatogram of patchouli alcohol extraction from *P. cablin* leaves.

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APPENDIX A

List of chemical used in the experiments.

No.	Items	Supplier
1	Nutrient (agar for microbiology)	MERCK
2	D-(-)-Fructose	SIGMA®
3	D-(+)-Glucose	SIGMA®
4	Gelrite	DUCHEFA
5	Sucrose	SIGMA
6	1-Naphthaleneacetic acid, Approx. 97%	SIGMA®
7	Sodium hydroxide	Duchefa Biochemie
8	2,4-Dichlorophenoxy	Duchefa Biochemie
9	Picloram	Duchefa Biochemie
10	6-Benzylaminopurine	SIGMA®
11	Murashige and Skoog Basal Medium with Gamborg's Vitamins	SIGMA®
12	Tween-20	
13	Hexane	Fisher scientific
14	Acetonitrile (HPLC grade)	Fisher scientific
15	Clorox	Bleech

APPENDIX B

Preparation of stocks solutions of Plant Growth Regulators:

Appendix B:

Method of preparing 1 mg/mL stock solution:

1) 100 mg or 0.1 g of plant growth regulator (PGR) was added into 50 ml distilled water.
2) 2-5 ml solvent (1M NaOH) was added to dissolve the powder.
3) The solution was stirred with magnetic stirrer while adding water to bring the final volume to 100ml.

Example: Add 1.0 ml of stock solution to 1 liter medium to acquire a final concentration of 1.0 mg/mL of the PGR in the medium.

FORMULA:

$$\text{Volume of stock solution} = \frac{\text{Desired PGR concentration x medium volume}}{\text{Stock solution concentration}}$$

Therefore, to prepare 1 liter of medium, the amount of PGR required are stated below:

PLANT GROWTH REGULATOR	DESIRED PGR CONCENTRATION	STOCK SOLUTION CONCENTRATION	VOLUME ADDED TO THE MEDIA
Picloram	1 mg/L	1 mg/L	1 mL
BA	1 mg/L	1 mg/L	1 mL
NAA	1 mg/L	1 mg/L	1 mL
2, 4-D	1 mg/L	1 mg/L	1 mL

APPENDIX C

C1: Callus induction

1 Litre distilled water

4.4 g/L Murashige and Skoog basal medium with Gamborg's vitamins

30 g/L sucrose

0.8 % w/v agar

3 mg/L picloram

C2: Callus growth and maintenance

1 Litre distilled water

4.4 g/L Murashige and Skoog basal medium with Gamborg's vitamins

30 g/L sucrose

0.8 % w/v agar

1 mg/L picloram

S: Suspension cultures growth and maintenance

1 Litre distilled water

4.4 g/L Murashige and Skoog basal medium with Gamborg's vitamins

30 g/L sucrose

1 mg/L picloram

PUBLICATION

INFLUENCE OF PLANT GROWTH REGULATORS AND LIGHT ON THE ESTABLISHMENT OF CALLUS AND CELL SUSPENSION CULTURES OF *POGOSTEMON CABLIN*

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Abstract

The young leaves from intact plant of *Pogostemon cablin* were used as the explants for callus culture induction. The effects of plant growth regulators (PGRs) and light were investigated on the growth of callus cultures. Callus cultures were established on the Murashige and Skoog (MS) media which were supplemented with sucrose (30 g/l), agar (8 g/l) and different concentrations and combinations of 2, 4-D, picloram and NAA. The induced callus cultures were incubated at 23-25°C under either continuous illumination, 16/8h photoperiod or in the dark condition. The best conditions for callus cultures induction were obtained in the medium containing 1 mg/L picloram which was maintained in the dark for 21 days. Callus cultures were healthy with white, yellowish and watery features. However, the cultured explants showed negative response to MS medium supplemented with 2, 4-D. The healthy, fast growing and friable callus cultures were then used to establish the cell suspension cultures. Under the influences of different concentrations and combinations of PGRs and light, rapid growth of cell suspension cultures were observed in the MS medium supplemented with 1 mg/L picloram and maintained in the dark.

Key words: MS- Murashige and Skoog, NAA- α-naphthalene acetic acid, Picloram, 2, 4-D - 2, 4-dichlorophenoxyacetic acid, Pogostemon cablin

INTRODUCTION

In recent years, interest in plant-derived pharmaceutical products and drugs has increased tremendously. For instance, the discoveries of antileukemic alkaloids vinblastine and vincristine from *Catharanthus roseus* [1], cytotoxic effects on human cancer cells from *Elaeagnus angustifolia* [2] and anticancer drug Taxol derived from *Taxus* sp. cell culture [3] have drawn attention of the researchers from all over the world to explore the prospect of other medicinal plants for their therapeutic abilities.

Plants also have always been the most prominent sources of carbohydrates, proteins, lipids as well as vitamins and medicines for the mankind. Over the millennia, plants also emerged as the valuable sources of a vast array of secondary metabolites used as flavours, fragrances, pigments, food additives, agrochemicals and pharmaceuticals [4]. Although most of the plants can be cultivated for the production of these fine chemicals, the levels are often very low in the plant, and hence the extracted products are usually very expensive. Some of the compounds are extracted from the wild plants and this method creates pressure on the natural resources [5]. 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.

Pogostemon cablin (Blanco) Benth (Syn. *Pogostemon* patchouli Hook) is part of the *Lamiaceae* family and is generally known as patchouli. It grows naturally in subtropical Himalaya, Far East and Southeast Asia. Besides that, P. cablin is also widely cultured in Malaysia, Indonesia, China and Brazil [6]. This oil can be used as anti-cancer agent to treat disease such as anti-influenza A (H2N2) virus [7], and also has other anti-microbial, anti-inflammatory, antibiotic, anti-microbial and anti-tumor properties [8]. Although there are some studies that had reported on callus induction of *P.cablin*, however the investigation on secondary metabolite production in callus or suspension cultures and patchouli alcohol level still very scarce and remain unfamiliar. From the previous study, a long period of time is needed to associate the metabolite difference in tissues by using morphogenic process [9].

In view of this, it is essential to establish the healthy growth of callus and cell suspension cultures as the source for the production of useful secondary metabolites. Various factors that influence the growth of callus and cell suspension cultures of *P. cablin* have been studied. The hormonal plant growth regulators (PGRs) usually have profound effects on both cell growth and secondary metabolite production [10]. By alteration of PGRs concentration, the growth of callus cultures of *Cupressus lusitanica* [11], *Orthosiphon stamineus* [12] and *Abrus precatorius* L. [13] were effectively improved. Light also plays an important role in these studies and has affected the production of secondary metabolites of nicotine and shikonin. However, light has no influence on the biosynthesis of anthraquinone and ubiquinone metabolites [14].

Consequently, the aim of this study was to investigate the effect of plant growth regulator and light on the establishment of callus and cell suspension of *P. cablin*. The best condition of growth of callus and cell suspension culture would affect the production of secondary metabolite especially the quality of the patchouli alcohol production.

METHOD

Callus culture induction

P. cablin plant was purchased from local plant nursery. The leaves were washed under running tap water and dried on tissue papers. The overall surface of the dried leaves was rubbed gently using cotton which was soaked with pure ethanol. Subsequently, the leaves were surface sterilized in 20 % (v/v) Clorox and three drops of Tween 20 for 15 minutes under continuous shaking. The leaves were then rinsed in sterilized distilled water under aseptic conditions at least five times to remove any traces of Clorox. After that, the leaves were cut into small squares (1cm x 1cm) and the explants were transferred aseptically and arranged onto the solid medium comprised of Murashige and Skoog (MS) basal medium, sucrose (30 g/L), agar (8 g/L) and picloram (3 mg/L) and pH of 5.6. This medium is designated as MS3. The explants were incubated in the dark at 23°C until the callus cultures were induced after 7-10 days of incubation.

Effect of PGR and light condition on callus culture maintenance

The callus cultures which were successfully induced were transferred in MS solid medium supplemented with 30 g/L sucrose, and different combinations and concentrations of picloram (P), 2, 4-dichlorophenoxyacetic acid (2, 4-D) and α-naphthalene acetic acid (NAA) between 0.25 to 3 mg/L. All the cultures were placed in culture room at 23°C maintained in three conditions; i.e. 24 hours under fluorescent light (1000 lux), 16/8 hour photoperiod and in the dark. The degree of callus formation in each medium was recorded weekly up to week 4. The observation was also made on the morphology of callus and type of callus formation induced.

Effect of PGR and light condition on cell suspension cultures

The best friable callus (10g) was transferred into liquid media supplemented with MS medium containing 30g/L sucrose and 1 mg/L picloram (the best concentration of PGR for maintenance of cell suspension cultures). The cells were subcultured after a week by adding 60ml fresh liquid media. To study the effect of PGR and light condition, cells from the two-week old inoculum were filtered. Every 2 g of cells were transferred into 20 ml liquid media with different concentration of picloram (P), NAA and 6-benzylaminopurine (BAP) and 2, 4-D among 0.5 to 1 mg/L, respectively. The different types of medium were labeled as shown in Table 1.

Table 1: Types of medium, light condition and PGRs concentration of cell suspension cultures.

Medium and light condition		Concentration of PGRs (mg/L)
Dark	Photoperiod	
MD1	MP1	1 P
MD2	MP2	1 BAP
MD3	MP3	1 NAA
MD4	MP4	1 2,4-D
MD5	MP5	1 2,4-D + 1 BAP
MD6	MP6	1 2, 4-D + 0.5 BAP

The cultures were maintained in the dark and 16/8 hour photoperiod at 23 °C under continuous shaking. The color and morphology of cell cultures were observed periodically.

Fresh weight (FW) and dry weight (DW) measurement

For each treatment, the content of flasks cultured was harvested as filtered biomass and dry weight was measured after drying the cells for 24 h at 75°C in the oven and weighed for each 3 days until 15 days.

RESULT AND DISCUSSIONS

Effect of PGRs and light on callus cultures

To maintain the best establishment of *P. cablin* callus culture, the effects of plant growth regulator (PGR) and light condition have been studied. Cytokinins and auxins are the PGRs that play major role in plant growth and development. There are two types of auxin which are naturally occurring and synthetic. IAA and IBA are identified as the naturally occurring auxin, while NAA, 2, 4-D, and picloram are the synthetic auxin. Three types of PGR were used in this studies, which are NAA (0.25-1 mg/L), 2, 4-D (0.25-1 mg/L), and picloram (1-3 mg/L). From the previous study, naturally auxin is less effective than synthetic auxin. Several types of auxin and cytokinin give dissimilar effects of callus formation process [15]. Most of the callus cultures turned to brown after two weeks when cultured in the medium either with or without PGR. From Table 2a,2b and 2c, it was observed that callus cultures grown on MS1 media and maintained in the dark was the best condition whereby callus was formed in a very short period of time (21 days) compared to other medium combination. This was followed by callus cultures grown in medium MS10 under all three light conditions. A significant amount of callus cultures was also formed in MS3 medium in the dark, MS8 medium under continuous light condition and MS9 medium under photoperiod although longer time was needed (30 days). Among all the treatment, callus cultures formed in MS1 medium were healthy, watery and friable with white and yellowish color. Nonetheless, in MS10 medium, the callus cultures produced were white, yellowish, watery and compact.

Table 2a: Influence of different concentration of picloram, 2, 4-D and NAA on the establishment of callus formation of *P. cablin* under photoperiod (16 hours light, 8 hours dark) condition

Media	Plant Growth Regulator (mg/L)			Callus colour/morphology	Degree of callus formation	Time (day)
	P	NAA	2,4,D			
MS 1	1			yellowish, greenish, watery	**	30
MS2	2	1		yellowish, watery	**	30
MS3	3			greenish, watery	***	30
MS4	1	0.25		dark brown, watery	*	30
MS5	1	0.75		yellowish, greenish, watery	**	30
MS6	1		0.25	brownish, watery	*	30
MS7	1		0.75	brownish, watery	**	30
MS8	2			greenish, watery	***	30
MS9	3	1		yellowish, watery	**	30
MS10		1		dark brown, watery	*	30
MS11	1	1		yellowish, greenish, watery	**	30
MS10		1		yellowish, white, greenish, watery	***	25
MS11	1	1		yellowish, greenish, watery	**	30
MS12	1		1	dark brown, watery	*	30

* Callus formed <1 cm; ** callus formed between 1.5-2 cm ;***callus formed more than 2.5 cm

Table 2b: Influence of different concentration of picloram, 2, 4-D and NAA on the establishment of callus formation of *P. cablin* under continuous light condition.

Media	Plant Growth Regulator (mg/L)			Callus colour/morphology	Degree of callus formation	Time (day)
	P	NAA	2,4,D			
MS1	1			white, yellowish, watery	***	21
MS2	2	1		greenish, watery	**	30
MS3	3			white, yellowish, watery	***	21
MS4	1	0.25		yellowish, greenish, watery	**	27
MS5	1	0.75		yellowish, greenish, watery	**	27
MS6	1		0.25	brownish, watery	*	27
MS7	1		0.75	dark brown	*	27
MS8	2			greenish, watery	**	30
MS9	3	1		greenish, watery	**	30
MS10		1		white, yellowish, watery, friable	***	25
MS11	1	1		yellowish, greenish, watery	**	27
MS12	1		1	brownish, watery	*	27

* Callus formed <1 cm; ** callus formed between 1.5-2 cm ;***callus formed more than 2.5 cm

Table 2c: Influence of different concentration of picloram, 2,4-D and NAA on the establishment of callus formation of *P. cablin* under dark condition.

Media	Plant Growth Regulator (mg/L)			Callus colour/morphology	Degree of callus formation	Time (day)
	P	NAA	2,4,D			
MS 1	1			yellowish, greenish, watery	**	30
MS2	2	1		brownish, yellowish, watery	**	29
MS3	3			greenish, watery	***	30
MS4	1	0.25		yellowish, greenish, watery	**	30
MS5	1	0.75		yellowish, greenish, watery	**	28
MS6	1		0.25	dark brown, watery	**	30
MS7	1		0.75	dark brown, watery	**	30
MS8	2			brownish, yellowish, watery	**	30
MS9	3	1		greenish, watery	***	29
MS10		1		yellowish, white, greenish, watery	***	25
MS11	1	1		yellowish, greenish, watery	**	30
MS12	1		1	dark brown, watery	*	28

*Callus formed <1 cm; ** callus formed between 1.5-2 cm ;***callus formed more than 2.5 cm

The different concentrations of PGR also give a considerable effect on callus formation process. However, the medium supplemented with different concentration of 2, 4-D (MS6, MS7, MS12) in all three light conditions showed the less callus cultures formation and the color of the callus turned to dark brown after 25 days. This observation is in line to the callus induction in Carob (*Ceratonia sliqua*) in which 2, 4 -D endorsed the formation of small callus which turned to brown and failed to develop. However, the low concentration of 2.4-D does not affect callus development [16]. Based on the observation, the callus maintained in the dark condition grew very well than the callus maintained under photoperiod or exposed to continuous light. The callus also became darker possibly because of the production and oxidation of phenolic

compounds from the explants [17]. Figure 1 shows the texture of the callus under dark, photoperiod and continuous light conditions, respectively.

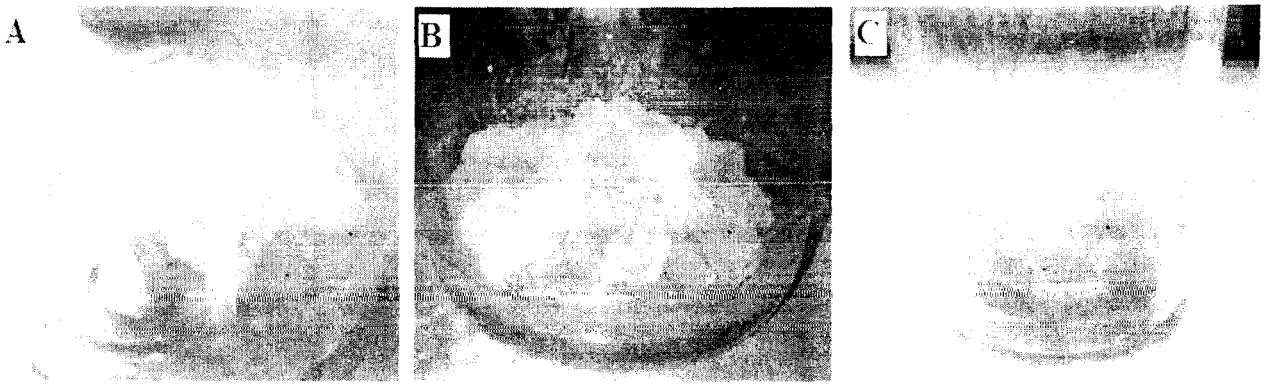

Figure 1: Callus formation from medium containing 1mg/L P and maintained in different condition: (A) dark, (B) photoperiod and (C) continuous light.

Effect of PGRs and light on *P. cablin* cell suspension cultures

The effect of PGRs and light has also been studied on the growth of *P. cablin* cell suspension cultures. The medium composition and physical factor such as light can has significant influence on the development of cell cultures [18]. In this section, the cell suspension cultures were grown in two different conditions, i.e. in the dark and photoperiod. This condition was chosen based on the observation from the result of callus cultures growth under continuous light, which was proved to be unfavourable for growth. From the growth profile, it was observed that all the cell lines displayed a sigmoidal curve. When the cell suspension cultures were grown in the dark condition (as shown in Figure 2 and Figure 3), cell suspension cultures from flask MD1 (12.03 g FW; 0.5 g DW) exhibited good growth rate and the color of the cells is light green and fine. This was followed by MD2 (11.06 g FW; 0.46g DW) but the cell showed a brown color, while MD3 (10.71 g FW; 0.41g DW) displayed the whitish color of cells. For other PGRs combination, the cells exhibited slow growth and produced a small amount of biomass (based on FW and DW measurement). The curve of suspension cultures trend in all mediums showed that the cells grew slowly at the first 3 days (lag phase) but increased exponentially at day 4 to day 12 (exponential phase) and reached stationary phase after day 15.

Meanwhile, cell suspension cultures that were maintained in photoperiod condition also showed a good growth profile, except MP5. Similar results were observed on growth and development of *Rollinia mucosa* cell suspension cultures, in which picloram was used as the plant growth regulator [19]. As depicted in Figure 4 and Figure 5, respectively, the exponential phase of MP1 is faster while the lag phase is shortest than others. However, a significant amount of cells was also obtained from MP2, MP3 and MP6 as indicated by fresh and dry weight in Figure 4 and Figure 5, although the lag phase is the longest. This is possibly because the cells were adapting to the new environment during the lag phase and started to consume the nutrients as they have adapted to the environment and eventually grew exponentially.

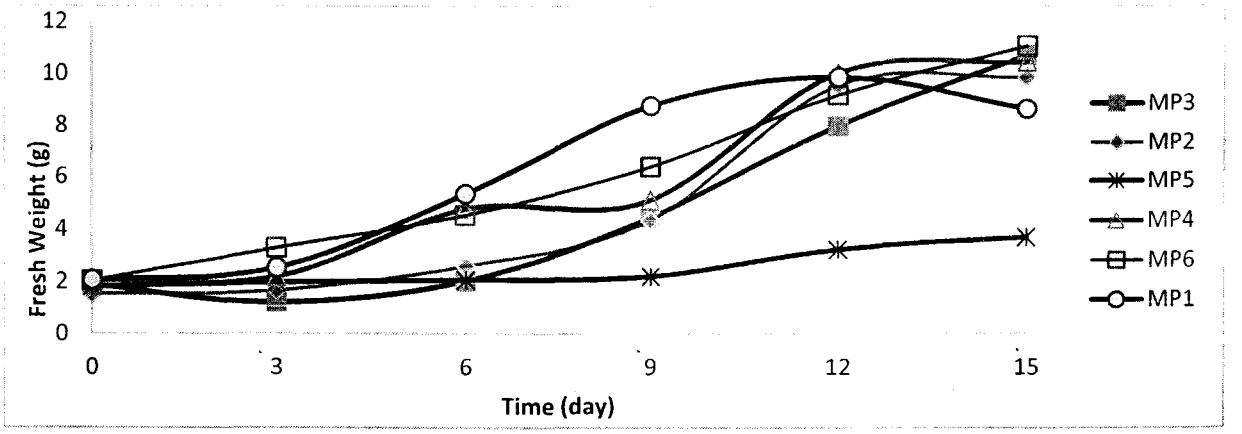

Figure 4: Fresh weight of cell suspension cultured for 15 days in photoperiod condition.

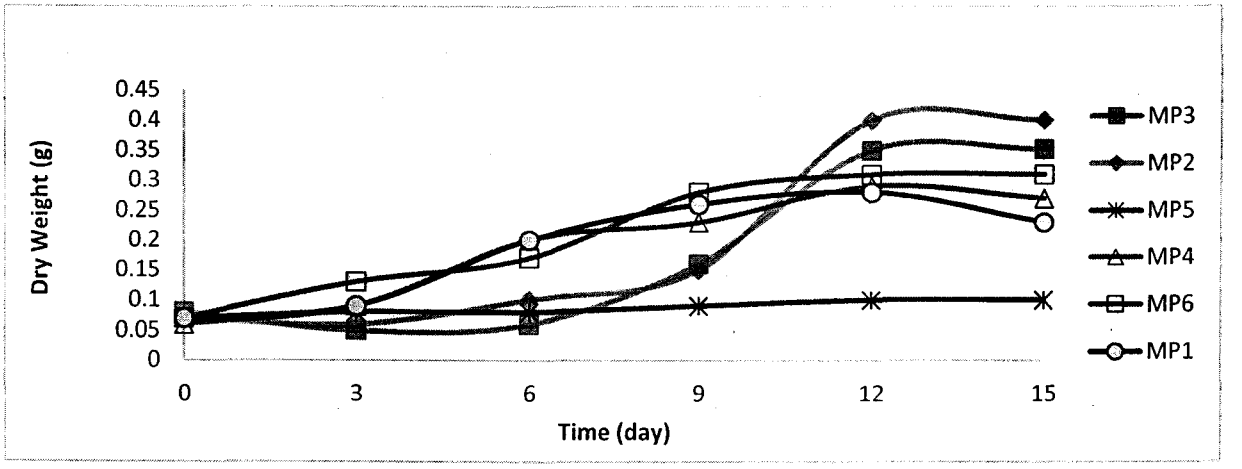

Figure 5: Dry weight of cell suspension culture for 15 days in photoperiod condition.

Table 3: Effect of different combination and concentrations of PGRs and light condition on the growth of *P. cablin* cell suspension cultures.

Condition	Medium	Colour of suspension cultures	Fresh weight (g)	Dry weight (g)
Dark	MD1	Whitish	12.03	0.50
	MD2	Brownish	11.06	0.46
	MD3	Whitish	10.71	0.41
	MD4	Brownish	8.89	0.30
	MD5	Light brown	5.80	0.14
	MD6	Yellowish	8.69	0.29
Photoperiod	MP1	Brownish	8.63	0.23
	MP2	Brownish	9.85	0.40
	MP3	Whitish	10.70	0.35
	MP4	Brownish	10.45	0.27
	MP5	Brownish	3.70	0.10
	MP6	Light green	11.05	0.31

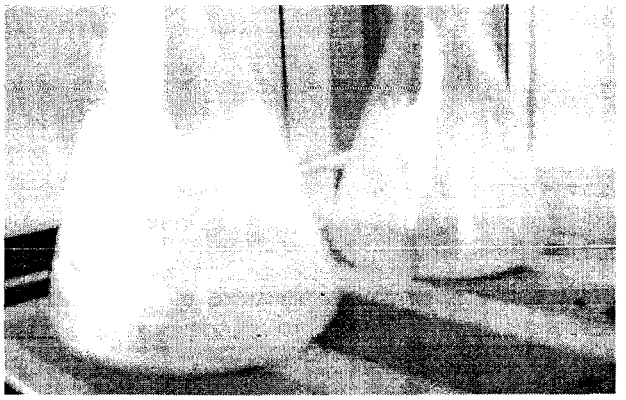

Figure 6: Cell suspension culture on medium containing 1mg/L picloram.

CONCLUSION

In the present studies, an establishment of rapid growing callus and cell suspension cultures of *P. cablin* was investigated particularly on the effects of PGRs and light conditions. It was revealed that *P. cablin* callus and cell suspension cultures could grow well in the medium containing 1 mg/L picloram and maintained in the dark condition. This result is useful for further studies on other the influencing parameters that could also contribute to the production of secondary metabolite, i.e. patchouli alcohol.

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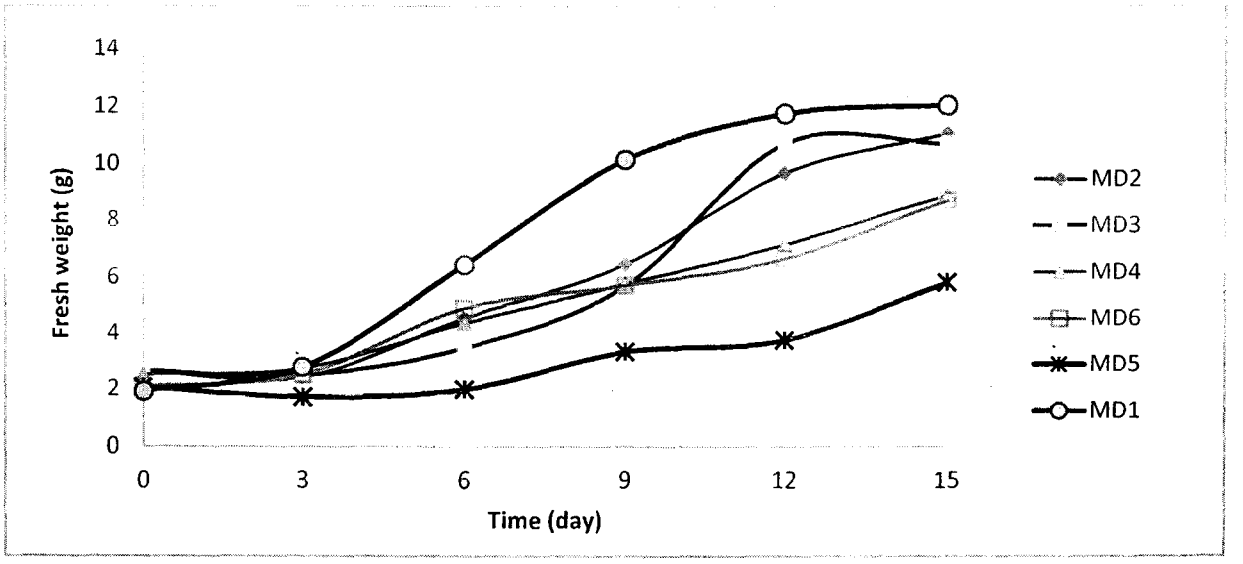

Figure 2: Fresh weight of cell suspension cultured for 15 days in dark condition.

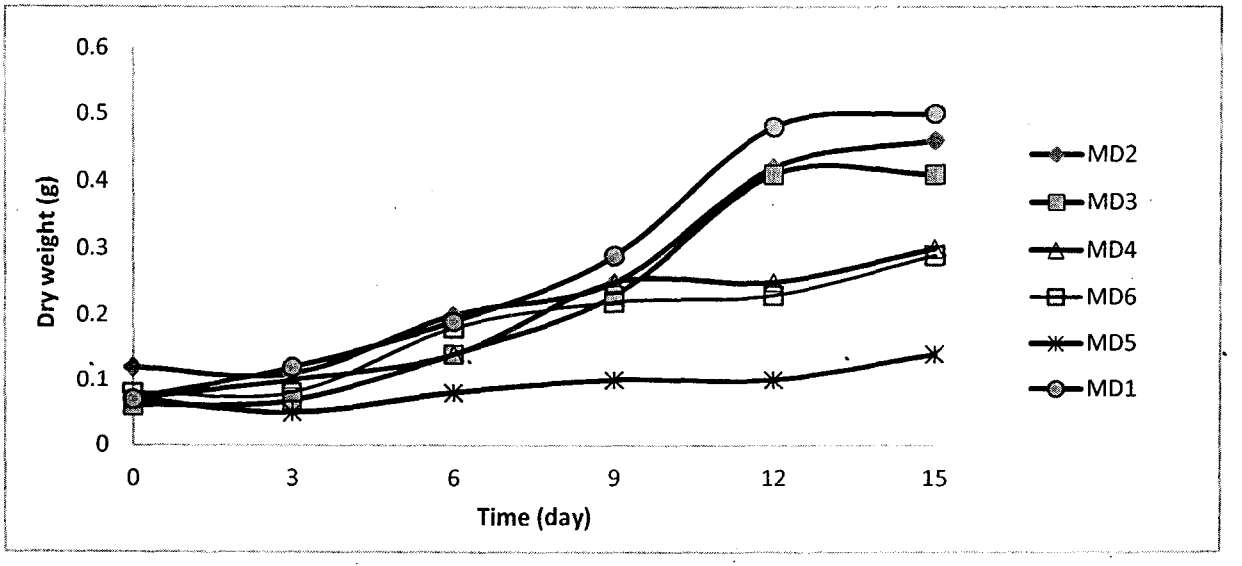

Figure 3: Dry weight of cell suspension cultured for 15 days in dark condition.

It was observed that the cells did not grow well in the medium containing the combination of 2, 4-D (1 mg/L) and BAP (1 mg/L), maintained under both light conditions (i.e. MD5 and MP5). However, when the cells were grown on these individual PGRs (information as given in Table 1), they exhibited good growth rate. This shows an inhibitory effect on the cell growth when these PGRs were supplied at equal amount, but promoted good cell growth when the concentration of BAP (cytokinin) was reduced by half, as obtained in flasks MD6 and MP6, respectively. Based on the summarized results as tabulated in Table 3, it was apparent that the supplementation of 1 mg/L picloram could promote good growth when the cell suspension cultures were grown in the dark condition (MD1) as compared to photoperiod (MP1).