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RESPONSE OF PLANT GROWTH REGULATORS ON THE GROWTH OF DIFFERENT TYPES OF IN VITRO CULTURES OF Pogostemon cablin (Blanco) BENTH FOR PATCHOULI ALCOHOL PRODUCTION

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Abstract

Patchouli Alcohol (PA) is one of the major active constituents in Pogostemon cablin (Blanco) Benth plant (P. cablin). The geographical, seasonal and environmental variations contribute to the fluctuation in oil content and quality. The plant cell culture technique has emerged as a substitute method for the production of essential oil. The present study was conducted to investigate the effect of Plant Growth Regulators (PGRs) on the growth and production of PA in vitro plantlets, callus as well as cell suspension cultures of P. cablin. Plantlets of P. cablin were successfully obtained by inoculating leaf explants on Murashige and Skoog (MS) medium augmented with α-Naphthalene Acetic Acid (NAA; 0.1 mg/L) and 6-Benzylaminopurine (BAP; 0.5 mg/L) as shooting hormones. Meanwhile, NAA (0.5 mg/L) was combined with activated charcoal (5 g/L) for rooting hormones. The effects of different types of auxin [Picloram, 2, 4-Dichlorophenoxyacetic acid (2, 4-D) and NAA] were investigated in order to determine the most suitable callus induction and maintenance medium. The best callus induction response was obtained on MS medium supplemented with 3 mg/L picloram in which, a healthy and friable callus was acquired in 21 days of culture. Callus was successfully maintained in MS medium added with a reduced concentration of picloram (1 mg/L). The results on the effect of PGRs in the cell suspension cultures of P. cablin indicated that picloram (1 mg/L) was the best growth hormone in initiating P. cablin cell suspension cultures in the dark condition. A higher dry cell weight (0.50 ± 0.03 g/20 mL) was produced in 15 days compared to the other PGRs used. Quantification of PA using gas chromatography with flame ionization detector confirmed that maximum yield of PA was obtained from the leaves of P. cablin plantlets (61.12 ± 1.39 mg/L), callus cultures (37.39 ± 1.19 mg/L) and cell suspension cultures (34.39 ± 1.26 mg/L). This study shows that picloram was the most suitable PGR for the growth of callus and cell suspension cultures as well as the production of PA.

Keywords: Callus cultures, Cell suspension cultures, Patchouli alcohol, Plant growth regulators, Pogostemon cablin.

1. Introduction

Pogostemon cablin (Blanco) Benth (P. cablin) or also known as patchouli is used worldwide as traditional medicine to remove dampness, stimulating appetite, relieving summer heat and also stopping vomiting [1]. P. cablin is classified under the lamiaceae family and it has been extensively cultivated in Malaysia, China as well as Brazil for its valuable oil namely patchouli oil [2]. Patchouli oil is a very volatile oil and has been used extensively in cosmetic and perfumery industries. It is also known that the major active ingredient in patchouli oil is Patchouli Alcohol (PA), a tricyclic sesquiterpene and the content of PA is usually used as an indicator to assess the quality of P. cablin [3].

Despite its potential in perfumery industries [4], a study on in vitro cultures of P. cablin plants was still scarce and very limited data were published. Yan et al. [5] reported the most recent study on in vitro cultures of P. cablin was on the initiation of 31 lines of octoploid plants using the colchicine-mediated technique. They confirmed that most of the octoploid plants produced exhibited a higher level of Patchouli Alcohol contents compared to the control group that was cultivated in 6 months [5].

According to Mathew and Sankar [6], plant cell culture technique has shown a higher rate of secondary metabolite formation and therefore, cell culture technology is advantageous for production of PA. Due to increasing demand of patchouli oil worldwide, various researches had been conducted in order to establish healthy and friable callus and cell suspension cultures as well as fast-growing in vitro plantlets of P. cablin. One of them is the selection of Plant Growth Regulators (PGRs). PGRs are also known as exogenous plant hormones and they are considered as a class of synthetic pesticides that have similarity in physiological activity with their natural hormone analogs [7].

The addition of suitable PGRs into plant cell culture medium will promote, inhibit or modify the physiological traits of plants and therefore maximize productivity as well as overcome limitations in plant productivity [8]. However, determination of PA content is not straightforward and conventional method has always failed to deliver accurate results [3]. Therefore, the objective of the current work was to investigate the response of PGRs on the growth of callus and cell suspension cultures along with subsequent analysis on PA content in vitro cell cultures including the plantlets using Gas Chromatography Flame Ionization Detector (GC-FID).

2. Materials and Method

2.1. Materials, reagent and chemicals

Dichloromethane (analytical reagent grade) was obtained from Merck (Darmstadt, Germany). Patchouli Alcohol, the reference standard with purity >98% was procured from ChemFaces, Wuhan, Hubei, China. PGRs used in this study such as 6-Benzylaminopurine (BAP), α-Naphthalene Acetic Acid (NAA) were both obtained from Sigma Aldrich while another two PGRs; 2, 4-Dichlorophenoxyacetic Acid (2, 4-D) and picloram were bought from Duchefa Biochemie. Chemicals used for pH adjustment; sodium hydroxide (NaOH) and hydrochloric acid (HCl) were bought from Merck and Fischer Scientific individually. Meanwhile, chemicals for medium preparation such as Murashige and Skoog (MS) basal medium, activated charcoal, and sucrose were all bought from

Sigma Aldrich while agar was bought from FC-Bios. Lastly, Tween-20 purchased from Duchefa Biochemie was used to sterilize the explants.

2.2. Plant material and culture conditions

Young leaves of P. cablin (Blanco) Benth was collected from local plant garden center in Malaysia. The leaves were surface sterilized by shaking in mixtures of 20% Clorox® with Tween-20 for 15 min, then cleansed thoroughly using distilled water (sterile). The explant that had been sterilized was later cut to 1 cm × 1 cm pieces using a sterile scalpel and forceps.

According to Virendra and Chawla [9], shooting and rooting media were prepared with slight modification in which, shooting hormones; 0.5 mg/L of BAP and 0.1 mg/L NAA were supplemented in MS medium with 30 g/L sucrose and 8 g/L agar to enhance multiple shoot formation. Rooting medium was prepared by adding 0.5 mg/L NAA and 5 g/L activated charcoal [10] into MS basal medium equipped with 30 g/L sucrose and 8 g/L agar for rooting enhancement. Both shooting and rooting media were adjusted to the pH of 5.75 ± 0.02 using 0.5 M NaOH and 0.5 M HCl prior to autoclaving at 121 ˚C for 15 minutes. The shooting medium was left to solidify and completely cooled down before 5 pieces of explants that had been cut into 1 cm x 1 cm pieces were transferred onto the medium. The cultures were then left to grow at the temperature of 25 ± 2 ˚C in 24 hours light condition for 30 days. As shoots emerged from the culture, they were then sliced off using a sterile scalpel and forceps and later was individually transferred onto the rooting medium for plantlets production. They were also left to grow for another 90 days at the same temperature and conditions as shooting medium. After 90 days of growth, the leaves from the plantlets were then harvested using a sterile scalpel and forceps.

The establishment of callus cultures was done by placing 5 pieces of 1 cm x 1 cm leaf explants on MS basal medium supplemented with 3% sucrose (w/v), 8% agar (w/v) and 3 mg/L picloram. The explants were incubated in the dark at the temperature of 25 ± 2 ˚C until the callus was induced in 7-10 days and were sub-cultured on the same medium for at least twice on 20 days interval. The friable callus cultures were then transferred to a maintenance medium containing 4.4 g/L MS basal medium, 8 g/L agar, 30 g/L sucrose and 1 mg/L picloram. The cultures were left to grow for 21 days and were sub-cultured for 2-3 times before the callus can be considered ready to be used in the experiment.

In order to study the effect of PGRs on the growth of P. cablin callus cultures, MS medium (4.4 g/L MS basal medium, 30 g/L sucrose, 8 g/L agar) containing various concentrations of PGRs (Table 1) were prepared accordingly. The pH of each medium was adjusted using 0.5 M NaOH and 0.5 M HCl to 5.75 ± 0.02 prior to autoclaving at 121 ˚C for 15 minutes. A pea size of callus cultures with a diameter of approximately 1 cm was placed onto the medium in the jar bottle using a sterile forceps. The cultures were cultivated in the dark for 30 days at 25 ± 2 ˚C and each set of experiment (MS1-MS17) was done in triplicate samples.

The changes in callus colour, growth and types of callus formed were then observed and recorded daily for a maximum of 30 days. The degree of callus formation was measured based on the diameter of the callus grown in the jar bottle and was represented as a symbol (*). Callus grown with a diameter of less than 1.5 cm were labelled as (*) and callus grown in between 1.5-2.0 cm diameter were

labelled as (**). Callus grown in between 2.0 cm to 2.5 cm were labelled as (***) and the best-grown callus in which, the diameter of callus formation was more than 2.5 cm were then labelled as (****).

Table 1. Combinations of PGRs used in the study for the growth of P. cablin callus cultures.

Media	Plant Growth Regulators (PGRs)		
	Picloram	NAA	2, 4-D
MS1	1	-	-
MS2	2	-	-
MS3	3	-	-
MS4	1	0.25	-
MS5	1	0.50	-
MS6	1	0.75	-
MS7	-	1	-
MS8	1	1	-
MS9	2	1	-
MS10	3	1	-
MS11	1	-	0.25
MS12	1	-	0.50
MS13	1	-	0.75
MS14	-	-	1
MS15	1	-	1
MS16	2	-	1
MS17	3	-	1

The cell suspension cultures were initiated using friable and healthy callus cultures that had been sub-cultured for at least five times. Suspension medium was prepared by adding MS basal medium (4.4 g/L), filtered sterile sucrose (30 g/L) and 1 mg/L picloram. The pH of the medium was adjusted using 0.5 *M* NaOH and 0.5 *M* HCl before autoclaving at 121 ˚C for 15 minutes. About 10% (w/v) of friable callus were then transferred into 100 mL of suspension medium in a 500 mL sterilized shake flask. The cultures were incubated in the dark at the temperature of 25 ± 2 ˚C on a rotary shaker and were shaken at 130 rpm for 3 weeks. The cell suspension cultures were sub-cultured every 7 days for 3 weeks by adding 60 mL of fresh liquid medium until it was intense enough to be used as the inoculum for the next experimentations.

In order to study the effects of PGRs in P. cablin cell suspension cultures, 2 g of fresh cells from the inoculum that were obtained by vacuum filtration were transferred into 20 mL liquid medium containing MS basal medium (4.4 g/L), 30 g/L sucrose (filter-sterilized) and supplied with various concentrations of PGRs (Table 2). The experiment was conducted for 15 days in triplicate to illustrate the trend of the cell growth and PA production. The samples were collected for every three days, and the fresh and dry cell weight were recorded.

Table 2. Combination of PGRs used in the study for the growth of P. cablin cell suspension cultures.

Medium	Concentrations of PGRs (mg/L)	Medium	Concentrations of PGRs (mg/L)
MD1	1 Picloram	**MD4**	1 2, 4-D
MD2	1 BAP	**MD5**	1 2, 4-D + 1 BAP
MD3	1 NAA	**MD6**	1 2, 4-D + 0.5 BAP

2.3. Patchouli Alcohol extraction and analysis

The collected samples from cell suspension and callus cultures that had been sub-cultured in MS medium containing the best concentration of PGRs for at least two times were preserved at -80 ˚C prior to freeze drying. The freeze-dried samples (0.4 g) were crushed into powder using mortar and pestle before addition of dichloromethane (16 mL). Extraction at room temperature was done in 72 hours. Extraction of plantlet was done by harvesting leaves from 90-days old plantlets. The leaves were oven dried at 40 ˚C for 24 hours. The drying method for in vitro plantlets was quite different compared to callus and CSC that were more fragile and heat sensitive. Although the vapour pressure of PA is low (25 ˚C), oven dried herbs (especially herbs from lamiaceae family) at 40 ˚C were proven to increase its total polyphenol content [11]. The dried leaves (0.4 g) were then crushed into powder and immersed in dichloromethane (16 mL) for 72 hours.

Quantification of PA was done based on Fan et al. [1] with slight modification. Gas chromatography (Agilent 7890A) set with flame ionization detector (FID) was used and PA was separated on a capillary column (HP-5: 30 m × 0.32 mm *i.d.*) covered with 0.25 µm film of 5% phenyl methyl siloxane. The temperature of the column was ignited to 100 ˚C for 1 minute, rose from 6 ˚C per minutes to 170 ˚C and held for 1 minute, then from 1 ˚C per minute to 180 ˚C. The splitless injection was employed using Helium (He) as a carrier gas (previously, the split injection was employed at the split ratio of 1:20 using nitrogen gas as carrier gas). Inlet and detector temperature were at 250 ˚C and 280 ˚C, respectively. The entire analytical operation was completed in 22 minutes (previously was done in 28 minutes). The standard solutions of PA were prepared from a stock solution and diluted to concentrations of 1, 2, 4, 32 and 64 µg/mL for GC analysis. The PA content in all extracts was determined using the standard calibration curve.

3. Results and Discussion

3.1. Effect of plant growth regulators in P. cablin callus and cell suspension cultures

The effects of different concentrations and combinations of three different PGRs in the class of auxin; picloram, NAA and 2, 4-D on the establishment of P. cablin callus cultures were given in Table 3. Cytokinin was not incorporated in this study as a possible PGR for the establishment of callus cultures because cytokinin usually plays an essential role in shoot organogenesis and not in the induction of callus [12]. The results were evaluated in terms of the day when the growth of callus at its maximum, the degree of callus formation (diameter) and morphology of the callus. From the data, it can be seen that each of the MS media that was supplemented with auxin as PGRs can successfully initiate the growth of callus. However, only a yellowish, watery and friable callus was considered healthy and preferable.

After four weeks of exposure to auxins, it was observed that the callus cultures were best grown in MS3 media (containing 3 mg/L) picloram) in which, the callus formed was yellowish and friable, the duration of the callus establishment was shorter (21 days) and the degree of callus formation was more than 2.5 cm in diameter. Ling et al. [13] supported finding that picloram with a concentration of 3 mg/L and above was the best in producing higher cells grow rate in ficus deltoidea callus cultures. However, past researchers have also reported on the different

combinations of PGRs to obtain the highest amount of callus cultures. This shows that the variation of PGRs used in the medium composition would affect the growth of callus cultures although the same plant species was used [2, 14].

Table 3. Influence of different concentration of plant growth regulators on the establishment of P. cablin callus cultures.

Media	Plant Growth Regulators (mg/L)			Callus color/ morphology	Degree of callus formation (diameter in cm)	Maximum growth of callus achieved (day)
	Picloram	NAA	2, 4-D			
MS1	1	-	-	Whitish, friable	***	21
MS2	2	-	-	Greenish, friable	**	30
MS3	3	-	-	Yellowish, friable	****	21
MS4	1	0.25	-	Yellowish, friable	**	27
MS5	1	0.50	-	Yellowish, friable	***	28
MS6	1	0.75	-	Yellowish, friable	**	27
MS7	-	1	-	Yellowish, compact	***	25
MS8	1	1	-	Yellowish, friable	**	27
MS9	2	1	-	Greenish, friable	**	30
MS10	3	1	-	Greenish, friable	**	30
MS11	1	-	0.25	Brownish, friable	*	27
MS12	1	-	0.50	Brownish, friable	***	30
MS13	1	-	0.75	Dark brown, friable	*	27
MS14	-	-	1	Brownish, friable	*	30
MS15	1	-	1	Brownish, friable	*	27
MS16	2	-	1	Brownish, friable	*	30
MS17	3	-	1	Brownish, friable	*	30

Diameter of callus formed: *callus formed <1.0 cm; **callus formed between 1.5-2.0 cm; ***callus formed between 2.0-2.5 cm; ****callus formed more than 2.5 cm.

Nonetheless, after the establishment of multiplying callus cultures, auxin may not be required for callus maintenance [15], although normally the maintenance medium has the exact composition of the medium for callus initiation. Therefore, the concentration of picloram was reduced to 1 mg/L for callus culture maintenance in this study. The initiation of cell suspension cultures from callus is very crucial since the suspension cultures contribute to an endless as well as a dependable source of natural products [16]. Hence, P. cablin suspension cultures were grown on MS medium supplemented with different concentrations of PGRs and the growth profile is as depicted in Fig 1.

It can be seen that the dry weight of P. cablin cell suspension cultures was the highest (0.50 ± 0.03 g/20 mL) dry weight) when 1 mg/L picloram was included in the medium (MD1), followed by MD2 medium (0.46 ± 0.01 g/20 mL) and MD3 medium (0.41 ± 0.04 g/20 mL). This shows that picloram is the best synthetic auxin used to increase the growth of P. cablin cell suspension cultures. Ahmad et al. [17] also reported the superiority of picloram over other PGRs in phyla nodiflora cell suspension cultures and this was mainly due to the fact that low concentration of picloram has stronger auxin activity. This property will thus promote a higher growth of plant tissues in vitro and accelerate the synthesis of new proteins [18]. In addition, it can also be seen from the data that inclusion of 1 mg/L BAP (synthetic cytokinin) into P. cablin cell suspension cultures also significantly increased the cell dry weight. According to Prins et al. [19], similar

finding was supported that the exogenous application of BAP in cell suspension cultures of mentha piperita has also increased the plant cells dry mass. Even though the addition of synthetic cytokinin was not required in the induction of callus (only high auxin concentration is needed to induce callus formation), its addition in cell suspension cultures is necessary for stimulating monoterpene accumulation [19, 20].

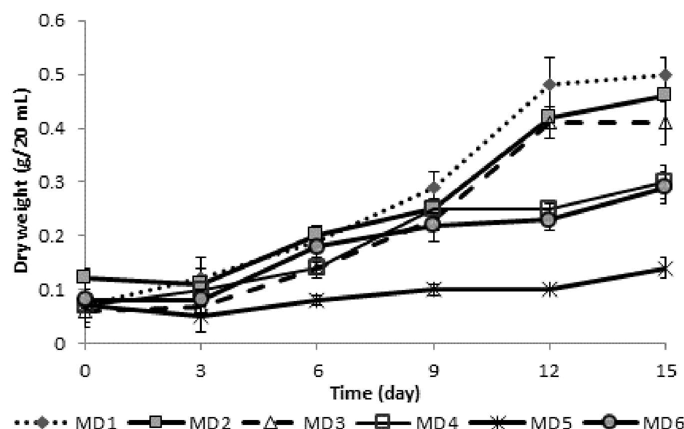

Fig. 1. Growth of P. cablin cell suspension cultures using different concentration of PGRs. [MD1: 1 mg/L picloram; MD2: 1 mg/L BAP; MD3: 1 mg/L NAA; MD4: 1mg/L 2, 4-D; MD5: 1 mg/L 2, 4-D + 1 BAP; MD6: 1 mg/L2, 4-D + 0.5 BAP. Values expressed as means SD (*n*= 3)].

3.2. Accumulation of Patchouli Alcohol in in vitro cultures of P. cablin

PA content was quantified in the three selected in vitro cultures. It was found that PA content was the highest in the young plantlets (3 months old) of P. cablin (61.12 ± 1.39 mg/L), followed by 21 days old callus (37.39 ± 1.19 mg/L) and 15 days old cell suspension cultures (34.39 ± 1.26 mg/L). Bunrathep et al. [2] supported the trend shows, that PA is a growth associated product. Interestingly, the PA content obtained from this study was higher than 19.9 mg/L for callus cultures grown on 0.5 mg/L NAA and 1 mg/L BAP. However, Santos et al. [14] could not detect PA in the callus cultures maintained in 0.113 mg/L BAP and 0.022 mg/L 2, 4-D. This shows that despite alteration of culture medium and growth conditions, callus and cell suspension cultures still failed to produce the desired secondary metabolite when they lack the specialized tissues or glands from the parent plants [21]. Although juvenile plantlets are the one producing a very high concentration of PA, it is believed that cell suspension cultures have the most immediate potential for industrial application. This is mainly because of its ability to be easily upscaled into a bigger volume in bioreactor thus ensuring the sustainability of the production system [22].

4. Conclusions

Plant cell cultivation is the best alternative for the manufacturing of essential compound. However, appropriate consideration must be given to the type and range of PGRs fed to the culture medium to boost up production of the targeted

compound(s). The results of this study demonstrate the superiority of picloram in establishing a healthy and friable callus as well as cell suspension cultures compared to 2, 4-D, NAA, and BAP. The protocol developed in this study is significant for future studies on cell growth and secondary product optimization of in vitro cultures of P. cablin.

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Abbreviations

2, 4-D	2, 4- Dichlorophenoxyacetic Acid
BAP	6-Benzylaminopurine
GC-FID	Gas Chromatography Flame Ionization Detector
He	Helium
MS	Murashige and Skoog
NAA	α-Naphthalene Acetic Acid
PA	Patchouli Alcohol
PGRs	Plant Growth Regulators

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