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Alkaloid Quinine in Cell Suspension Culture of Cinchona Ledgeriana Moens: Biological Study towards Industrial Application

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Abstract. Alkaloid quinine has many benefits in medicinal as well as in food industries. Salt of quinine has been produced as one of the pharmaceutical industries in Indonesia. Lack of raw material that is cinchona bark is a major problem and consequently increase the production cost. Imported cinchona bark from several African countries contains very low level of quinine. Cell suspension cultures of Cinchona ledgeriana clone QRC 315 exhibited a high percentage of quinine, 7 to 11%. The cells were treated with plant growth retardants abscisic acid (ABA) or paclobutrazol (PBZ), combined with sucrose as a source of carbohydrate or with mannitol as a partial substitution of sucrose. Double applications of stress-inducing agent, 7 mg/L PBZ and mannitol resulted in 10.9% quinine, the highest level, but with the least proliferated cells. Addition of 3 mg/L ABA combined with sucrose presented only 6.8% of quinine but with the highest production of cell biomass that eventually gave substantial amount of total quinine. With some more improvements on the treatments applied to the cell suspension culture, the technique may be developed from laboratory to industry through the use of bioreactors.

Keywords: quinine, Cinchona ledgeriana, cell suspension culture, industry

1. Introduction

The world demand for alkaloid quinine tends to increase. The substance is useful to prevent and to treat malaria in human being. The World Health Organization [1] reported that in 2013, there were around 198 million (ranged 124–283 million) cases of malaria worldwide, and an estimated 584 000 (ranged 367 000–755 000) deaths. Ninety percent of all malaria deaths occur in Africa. Some parts of Indonesia are still endemic malaria; around 1.8 million cases were reported. Among the different kinds of drugs for treating malaria disease, quinine is the most frequently used. This alkaloid is also known for other purposes such as analgesic and anti-inflammatory agent, as remedy for nocturnal leg cramp, ingredient in certain cosmetics, and as food additive to give a bitter taste in soft drinks [2]. Demand for quinine to supply food and cosmetic industries is substantially greater.

Franz Wilhelm Junghuhn introduced quinine producing plant named *Cinchona* spp. to Indonesia in 1852 and subsequently large cinchona plantations were established at highlands of West Java. The first and the biggest pharmaceutical industry in Indonesia had been built in Bandung, the capital city of West Java Province. The problem raised when people including the government who own cinchona plantations have abandoned cinchona plants and shifted to other more profitable crop plants, such as tea, coffee, and horticultural crops. Moreover, the rest of the plants are not productive anymore due to their age [3]. A huge

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quantity of cinchona bark for the processing industry of alkaloids must be imported from Kenya and Congo[1]. The quinine content of the african cinchona bark, however, is quite low, only 5 to 5.8%, while the Indonesian cinchona has 7 to 8%. It means that Indonesians have brought woody waste from Africa. This situation is far from cost-benefit and even not environmentally friendly.

Plant cell cultures have been developed to produce secondary metabolites, among them was cinchona cells [4]-[7]. Precursors and many stress-inducing agents have been applied in plant cell culture to induce the synthesis of certain secondary metabolites; some were successful and some other failed due to many factors. Periderm of cinchona bark contains the best level of alkaloid quinine compared to other parts of the plant, but also its cultured cells is capable to synthesize the alkaloid. These studies aimed to observe the effects of some stress-inducing agents on the capacity of cinchona cells to synthesize quinine. The ultimate objective is to supply industries with quinine independently on season, plant growth or the availability of planting areas.

2. Materials and Methods

2.1 Initiation of cell suspension culture and treatments

Friable calli was collected from *in vitro* cultures described in Ratnadewi and Sumaryono [8] to initiate cell suspension cultures. In the liquid media, various stress-inducing agents were applied, from the beginning of the culture or five weeks afterward. The stress-inducing agents used were abscisic acid (ABA 1 and 3 mg/L), paclobutrazol (PBZ 1, 3, 5 and 7 mg/L) which were combined with normal concentration (30 g/L) of sucrose or with mannitol as partial substitution to sucrose. The basic media in which the treatments applied was described elsewhere [9].

In the first work where Treatments I were applied on clone QRC 313, the data of quinine determination were used as comparison to clone QRC 315. With the aim to improve the level of quinine content, clone QRC 315 was used in the second work (Treatments II).

The cell cultures were agitated on an orbital shaker at 100 rpm, in room temperature of 25 ^{o}C and under light intensity of 10 µmol/m^2/sec for 16 hours a day. Cell volume after sedimentation (CVS), a non-destructive method, was used to measure the cell growth. Cell volume in milliliter was then calibrated into gram through the formula {(*a* 0.4565) 0.3252}, where *a* corresponds to volume in mL.

2.2 Quinine extraction and HPLC analysis

At the seventh week of culture, the cells were harvested for quinine determination, following the cell growth rate reported by Sumaryono and Riyadi [10]. Reversed-phase high performance liquid chromatography (HPLC; Varian-USA, type 940) was used to measure the major alkaloid quinine.

Quinoline extraction and analysis was performed as described by Klink [11], with some modifications. Some oven-dried cell powder (0.5 g) was boiled in 95 mL aquadest for 25 min, then allowed it to cool at room temperature. The liquid was then adjusted to 100 mL by adding aquadest. A 5-mL aliquot was taken from decanted solution and filtered through a 0.45-µm millipore filter. The filtered liquid was injected into an HPLC column (Pursuit XRs 3 µ C-18, column length 150 cm x 4.6 mm). The column temperature was adjusted to 30 ^{o}C. Aquadest, acetonitrile, and glacial acetic acid (ratio 81:18:1, respectively) served as an eluent, with a 0.6 mL per minute flow rate and six attenuations. Standard quinine alkaloid (Sigma, Germany) served as positive control. The chromatogram was detected by UV-Vis at 250 nm. [1]

3. Results and Discussion

3.1 Cell growth in suspension culture

The cell growth increased exponentially from the first week up to the fourth week and subsequently slowed down till the sixth week, then started to decrease thereafter (Fig. 1).

ABA at 1 and 3 mg/L in medium with normal source of sugar (sucrose 30 g/L) demonstrated a growth promotion compared to the untreated cells (Control). At the time of harvesting at week 7, they reached 7 to 9 g fresh weight per flask. Contrary to ABA, PBZ at 5 mg/L combined with 5.3 g/L mannitol and 20 g/L

[1]Personal communication with a staff of PT Sinkona Indonesia Lestari, Subang-West Java.

sucrose decreased cell growth and at 7 mg/L the cells was remarkably suppressed (2 and 4 g fresh weight per flask, respectively). Rademacher [12] and Chaney [13] suggested that PBZ reduces the rate of cell division which is expressed mainly by reducing internodal growth, without causing any toxicity. Cell division and differentiation still occur but at a lower rate [13]. It inhibits the oxydation of kaurene into kaurenoic acid in the biosynthesis of gibberellin [14]. This would explain partial role of PBZ in reducing cell growth or plant growth.

Growth regulators are pleiotropic; one regulator has several effects in plant. Both ABA and PBZ are known as growth promotors as well as growth retardants. As growth retardants, both ABA and PBZ induce plants to enable to withstand harsher environmental conditions [13], among others by reducing growth or inducing dormancy.

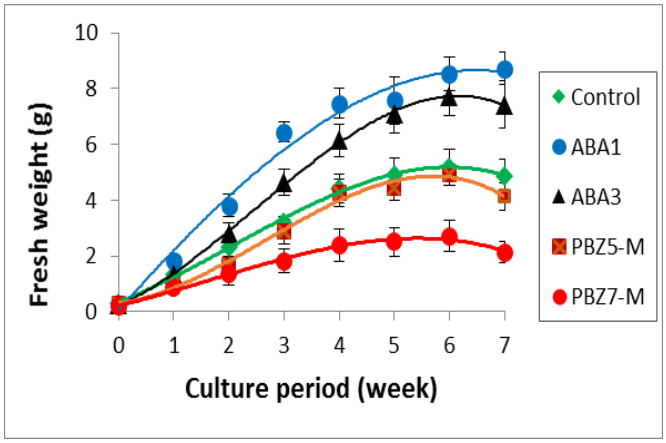

Fig. 1: Cell growth rates of clone QRC 315 affected by ABA and PBZ.

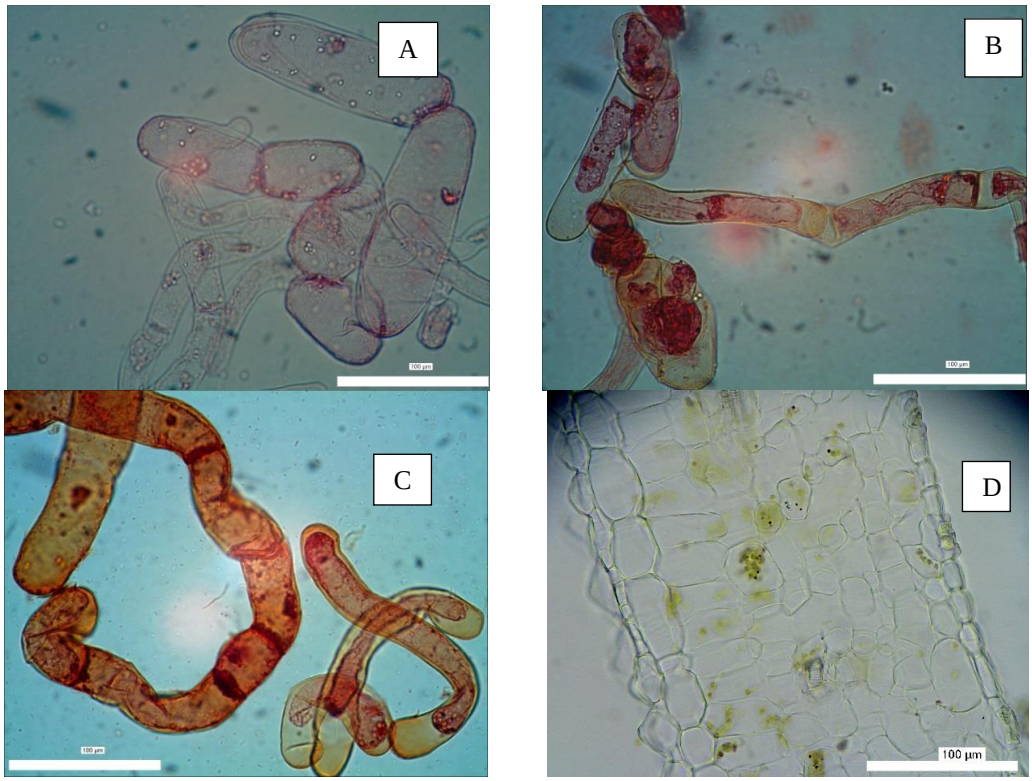

Fig 2: Elongated cells of C. ledgeriana Moens QRC 315 treated with ABA and PBZ, observed 7 weeks of culture age.
A). Effect of 3 mg/L ABA (ABA 3); B). Effect of 7 mg/L PBZ + mannitol (PBZ 7-M); C). Control cells;
D). C. ledgeriana cells in leaf.

Mannitol was incorporated into the media as an osmotic stress-inducing agent, with aimed to give additional stress to the cells. PBZ in normal media (with 3% sucrose) suppressed cell growth [8]. Therefore,

the data presented here indicated that mannitol indeed created another stress on the cells in addition to that caused by PBZ. Different to sucrose, mannitol, a sugar alcohol, cannot be metabolized by plant cells. Stoop and Pharr [15] reported that in celery suspension cultures, mannitol was taken up by the cells in high concentration in contrast with the uptake of sucrose, fructose, glucose, and mannose which were extremely low. It was suggested that the limited uptake of sucrose might happen when mannitol was added to the media which gave more suppressive conditions to cinchona cells. Fig. 2 reconfirmed that phenomenon: the majority of cells treated with 3 mg/L ABA were still vigorous indicated by the intact cytoplasm (Fig. 2 A). It is similar to the untreated cells (Control, Fig. 2 C). However, cells treated with PBZ and mannitol were plasmolyzed (Fig. 2 B). Fig. 2 indicates also that the structure of cultured cells is modified to long cells which is much different to the original cells in leaf (Fig. 2 D).

3.2 Quinine in Cinchona cultured cells

In the first work (treatments I), 30 g/L sucrose was used as a sole source of sugar in the cell suspension media. Table 1 shows that when ABA or PBZ was incorporated into the media at the fifth week of culture, the production of quinine was not so much increased compared to the Control in case of ABA 1-5 and ABA 3-5. Meanwhile PBZ 1-5, 3-5 and 5-5 respectively produced increasing level of quinine. This facts show that PBZ is stronger than ABA as stress-inducing agent. When PBZ was added to the media from the initial culture, the quinine content augmented 10 to 11 folds higher than that of the Control cells. It is presumed that to create a stress, growth retardants need a longer time to effect. Therefore, the treatments applied to the cells had been improved in the following work.

In the second work (Treatments II), another promising clone of *C. ledgeriana* QRC 315 was used. ABA and PBZ were added in the culture media from the starting point. As much as 5.3 g/L of mannitol was used in addition to 20 g/L sucrose. The molarity of 5.3 g/L mannitol is equivalent to 10 g/L sucrose that makes it equal to 30 g/L sucrose. The clone cell culture exhibited much higher content of quinine. It was demonstrated by the Control cells as well that gave 7.12% quinine level. ABA 1, ABA 3 and PBZ 5-M produced comparable amount of quinine to the untreated cells. PBZ 7-M gave the highest percentage of quinine. These data suggested that clone QRC 315 is remarkably superior to clone QRC 313, and by the application of double stress-inducing agents which were PBZ 7 mg/L and mannitol as partial substitution of sucrose, cinchona cells were promoted to synthesize more quinine (10.9%). It is particularly important because this percentage is much better than that extracted from cinchona bark cultivated in the country or imported from Africa.

Table 1: Quinine content in cultured cells of cinchona treated with ABA and PBZ

Clone QRC 313		Clone QRC 315	
Treatments I	Quinine (% DW)	Treatments II	Quinine (% DW)
Control	0.011	Control	7.12
ABA 1-5	0.015	ABA 1	5.70
ABA 3-5	0.016	ABA 3	6.83
PBZ 1	0.121	PBZ 5-M	5.75
PBZ 3	0.113	PBZ 7-M	10.90
PBZ 5	0.002		
PBZ 1-5	0.021		
PBZ 3-5	0.066		
PBZ 5-5	0.087		

Note: - Analyzed at the seventh week of culture.
- All values shown are means of duplicate samples.
- PBZ 5-M and PBZ 7-M mean respectively 5 mg/L and 7 mg/L of PBZ plus mannitol and sucrose.

The percentages of quinine would not mean much when the cells biomass is not abundant enough that in turn would not provide expected amount of quinine for commercial purposes. When we correlated the cell growth and the quinine content (Table 2), we realized that ABA at 3 mg/L with normal level of sucrose (ABA 3) gave the best amount of cells. With only 6.83 % of quinine, ABA 3 provided the most abundant of

quinine. Meanwhile PBZ 7-M provided the smallest amount of biomass, despite the important percentage (10.9 %) of quinine that resulted in the smallest amount of total quinine.

Table 2: Total amount of quinine produced per flask*

Treatments II (Clone QRC 315)	Means of cell dry weight/flask (g)	Quinine (%)	Total quinine per flask (mg)	Estimation of total quinine in 1000 L media in a bioreactor (g)
Control	0.115 ± 0.045	7.12	8.19	409.5
ABA 1	0.175 ± 0.032	5.70	9.97	498.5
ABA 3	0.206 ± 0.043	6.83	14.07	703.5
PBZ 5-M	0.097 ± 0.064	5.75	5.58	279.0
PBZ 7-M	0.050 ± 0.037	10.90	5.45	272.5

* The flask contained 20 mL of media; the cells were harvested after 7 weeks of culture

From the research, we are reconfirmed that the clone QRC 315 of C. ledgeriana is superior to the clone QRC 313. Those results challenge us to search for better methods to improve cell proliferation in the suspension culture in one point while maintaining high percentage of quinine produced by the cells in another point.

Barrett [16] as well as Kurian and Sankar [17] mentioned that in general the first harvest of cinchona bark can be executed after 6 to 12 years, and it may be repeated every following 3 to 5 years, while from cell suspension culture quinine can be collected every seven weeks. Therefore, cell suspension culture is a promising technology in quinine production for industrial scale in the near future. Lab cultures in flasks can be shifted into bioreactors.

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