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Quinoline Alkaloids in Suspension Cultures of *Cinchona ledgeriana* Treated with Various Substances

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Cinchona alkaloids are in extensive uses, not only for drugs but also for soft drink industries. They are harvested from the bark of trees *Cinchona* spp. after certain ages and therefore are available over a limited time. Cell culture is an alternative way to continuously produce such secondary metabolites in a much shorter time. Various substances were added in the normal growth media to promote quinoline alkaloids production by cell cultures of *Cinchona ledgeriana*. At the sixth week of culture, quinine and cinchonine contents were suppressed by paclobutrazol (PBZ), abscisic acid (ABA), or even by precursor tryptophan, while cinchonidine content was enhanced by 0.2 mg/l tryptophan to 43 fold of that produced by untreated cells (2.8% dry weight). At the seventh week of culture, the production of quinine and quinidine started to grow whereas the production of cinchonine and cinchonidine tended to decrease. An addition of 5 mg/l PBZ to culture media yielded the highest level of total quinine/quinidine after seven weeks, e.g. quinine 11 times more abundant and quinidine 23 fold higher compared to the untreated cells. Particularly the level of quinine which is the most demanded for medical and industrial purposes still need to be improved to approach to or even higher than that of extracted from the conventional source.

Key words: alkaloids, paclobutrazol, abscisic acid, tryptophan, cell suspension culture, *Cinchona ledgeriana*

INTRODUCTION

Cinchona bark contains quinoline alkaloids. Quinine, quinidine, cinchonine, and cinchonidine are the major substances among over thirty others (McCalley 2002). The demand for quinine is increasing due to their extensive uses as antimalaria and also as ingredient in the preparation for treatments of colds, cough, influenza, and various fevers. In addition to its drug values, quinine is substantially used in the manufacture of tonic drinks. The salts of quinine are also added to hair oils, sunburn lotions, moth repellents and insecticides. Quinidine is known as a remedy against cardiac ailments. Cinchonidine, having weaker action than quinine, is useful as an antispasmodic in whooping cough (Peter *et al*. 2007). The presence of cinchonine in a mixture of quinine and quinidine was proven to be more effective against quinine-resistant strains of *Plasmodium falciparum* (Druilhe *et al*. 1988).

Production of cinchona alkaloids in cell suspension cultures and its enhancement by using stress, precursors, elicitors, and the use of hairy roots system have been reported (Wijnsma *et al*. 1986; Hamill *et al.* 1989; Toruan-Mathius *et al*. 2006). By treating leaf, shoot, and organ cultures of *C. ledgeriana* with benzyladenin in Murashige-Skoog (MS) media, the content of alkaloids augmented with the increase in cultures age. Thirty two-week-old tissue cultures contained the same amount of alkaloids as one-year-old plant. Feeding various precursors to eight-week-old leaf shoot cultures increased the total alkaloids content by 66% with tryptophan, 42% with secologanin, and 5% with strictosidine-type alkaloid intermediates (Peter *et al.* 2007).

The aim of this research work was to improve the content of quinoline alkaloids, particularly quinine, in cell suspension cultures. In addition to that, the influence of various substances incorporated in the media to the cells' alkaloids production was also investigated.

MATERIALS AND METHODS

Plant Material. Fast growing callus initiated from leaves of axenic seedlings of *C. ledgeriana* was used as material source for suspension cultures. Cells were harvested after two weeks of homogenization in the baffle flasks (Sumaryono & Riyadi 2005). Approximately one gram of cells filtered through a mesh of 500 µm were transferred into 20 ml of basic WP medium (Lloyd & McCown 1981) containing 30 g/l sucrose, 1 µM phloroglucinol, 15 µM picloram, and 0.5 µM benzyladenin (BA), added with certain substances as treatments.

Treatments of Cell Cultures. Treatments with growth retardants paclobutrazol (PBZ: 1, 3, and 5 mg/l, annotated as PBZ 1, 3, and 5) and a precursor for quinoline synthesis, tryptophan, at 0.2 and 2 mg/l (Tryp 02 and Tryp 2) were applied to enhance the production of quinoline, from the beginning of the culture. This constituted the starting

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point of treatments on the cell cultures. Some other cell cultures were instead exposed to 1, 3, and 5 mg/l of PBZ or abscisic acid (ABA) 1 and 3 mg/l at the fifth week (annotated as PBZ 1-5, 3-5, 5-5, and ABA 1-5, 3-5). Ten flasks represented each treatment.

The treated cell suspension cultures and controls were agitated on an orbital shaker at 100 rpm, under the light intensity of 10 µmol/m^2/sec. and temperature of 25 °C. The cell growth rate was measured by CVS (cell volume after sedimentation) method every week up to the harvesting day.

Alkaloid Extraction and Analysis. The cells were collected from five flasks through filtration, at the sixth and the seventh week for quinoline analysis. The levels of quinoline, i.e. quinine, quinidine, cinchonine, and cinchonidine were determined.

For extraction and purification, 0.5 g oven-dried cells powder was boiled in 95 ml aquadest. Five milliliters aliquot were taken from the decanted solution, filtered through Millipore 0.45 µm, and 5 µl of it was injected into the HPLC column (Pursuit XRs 3 µ C_{18}, column length 150 cm X 4.6 mm id), and performed at 30 °C. Quinoline standards were used for determination. The eluent was the mixture of water:acetonitrile:glacial acetic acid = 81:18:1. The flow rate was adjusted to 0.6 ml/min. and the attenuation was 6. UV-Vis 250 nm was employed as detector (Klink 1979). Data from each treatment and age of culture were average of duplo determinations.

RESULTS

The Growth of Treated Suspension Cell Cultures. The cell growth started to enhance remarkably in all cultures at the third week, and it continued to increase until the sixth week. The application of PBZ 1 at the beginning of the treatment did not reduce the growth capacity but higher levels of PBZ (PBZ 3 and 5) lowered their growth (Figure 1a), with PBZ 5 affected more significantly. Contrary to that, the application of the same growth retardant to the cells after letting them grow in basic media for five weeks (PBZ 1-5, 3-5, 5-5) gave no negative effects to the cells (Figure 1b); there was even a tendency to promote. The use of ABA in the cultures five weeks later (ABA 1-5 and 3-5) also demonstrated cell growth enhancement.

Tryptophan is a precursor in the synthesis pathway of quinoline alkaloid. Tryptophan feeding into the culture media maintained the growth rate of cinchona cells although it was lower than that of the untreated ones (Figure 1c).

The Alkaloids Content. Quinoline is alkaloids of *Cinchona*, secondary metabolites which have been proven being synthesized by cultured cells *in vitro* (Payne *et al*. 1987; Robins *et al*. 1987). Table 1 demonstrated that the untreated cells also produced quinoline after six weeks of culture. Supply of growth retardants PBZ and ABA as well as precursor of alkaloids L-tryptophan reduced the synthesis of quinine and mostly of cinchonine. Quinidine was not detected in all treatments at the sixth week of

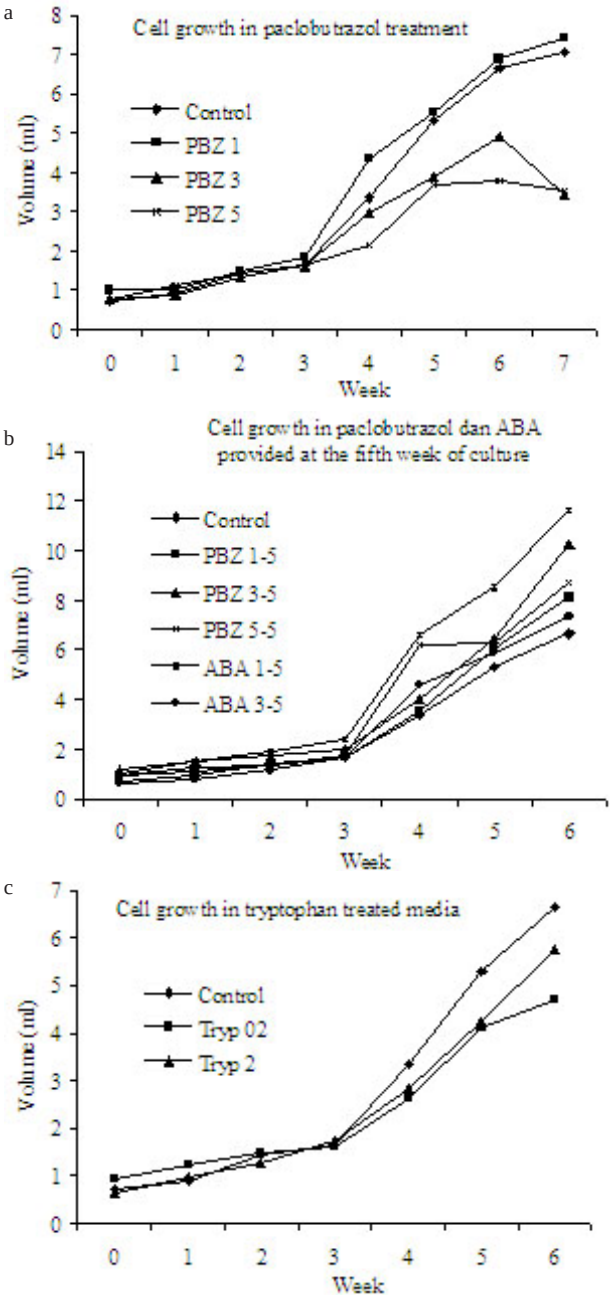

Figure 1. The growth curves of cells in media treated with various substances: a: PBZ; b: PBZ; and ABA supplied after five weeks of culture; c: Tryptophan. Means of ten flasks of cell cultures per treatment.

culture. In contrast to those results, the production of cinchonidine was favored by Tryp 02 and Tryp 2 as well as by PBZ 1 incorporated one week before analysis (PBZ 1-5); the other treatments resulted in lower content of cinchonidine.

At the seventh week of culture, production of quinine and particularly quinidine remarkably increased from the sixth week state. However, cinchonidine drastically dropped compared to one week before. Cinchonine appeared only at the seventh week from the treatments PBZ 1, PBZ 3, both levels of tryptophan, and PBZ 1-5 while none of PBZ 5, PBZ 3-5, and ABA 3-5 gave

Table 1. Quinoline content (µg/g) in *C. ledgeriana* cell cultures treated with various substances

Treatment	Quinine		Quinidine		Chinchonine		Chinchonidine	
	Wk-6	Wk-7	Wk-6	Wk-7	Wk-6	Wk-7	Wk-6	Wk-7
Control	72.58	108.38	ND	349.38	104.84	ND	649.22	136.56
PBZ 1	38.30	151.06	ND	510.58	ND	51.32	24.04	115.52
PBZ 3	19.84	158.90	ND	522.74	ND	132.00	97.12	133.28
PBZ 5	18.96	1206.70	ND	8078.44	ND	ND	50.68	243.28
Tryp 02	ND	57.38	ND	32.20	ND	97.92	27864.70	10.20
Tryp 2	14.70	875.48	ND	899.22	ND	21.38	799.36	17.58
PBZ 1-5	62.80	1129.24	ND	2771.68	ND	129.72	3421.80	56.08
PBZ 3-5	50.86	15.36	ND	ND	ND	ND	69.46	12.52
PBZ 5-5	65.34	208.48	ND	234.68	83.76	ND	21.22	12.24
ABA 1-5	45.06	663.40	ND	290.32	114.38	ND	256.14	25.34
ABA 3-5	28.60	872.32	ND	277.38	ND	ND	25.88	20.98

ND = Not detected.

Table 2. Total quinoline produced in cell cultures after six and seven weeks of culture

Treatment	Total quinoline (%)	
	Wk-6	Wk-7
Control	0.080	0.060
PBZ 1	0.006	0.080
PBZ 3	0.010	0.090
PBZ 5	0.007	0.950
Tryp 02	2.800	0.020
Tryp 2	0.080	0.180
PBZ 1-5	0.350	0.410
PBZ 3-5	0.010	0.003
PBZ 5-5	0.020	0.040
ABA 1-5	0.040	0.100
ABA 3-5	0.005	0.120

cinchonine. In other treatments, cinchonine produced at the sixth week was no longer detected one week later.

In general, PBZ 5 produced the highest level of quinine (1,206 µg/g or 0.12%) and quinidine (8,078 µg/g or 0.81%) at the seventh week, while PBZ 1-5 gave good levels of both substances, and cinchonidine one week earlier. Tryp 02 resulted in the best production of cinchonidine (27,864 µg/g) which is equivalent to 2.8% or 43-fold as much as the control one, at the sixth week. Tryp 2 was moderately good in promoting the synthesis of quinine and quinidine at the seventh week and cinchonidine at the sixth week. ABA showed little enhancing effect on quinine production.

Quinine is stereoisomer to quinidine and cinchonine is stereoisomer to cinchonidine. The results demonstrated that in *C. ledgeriana*, quinine was synthesized earlier than quinidine, and cinchonidine was produced in advance and much more dominant than cinchonine. Regarding to the total four alkaloid substances, quinine, quinidine, cinchonine, and cinchonidine, the highest total alkaloids was provided by Tryp 02 at the sixth week and by PBZ 5 at the seventh week (Table 2).

DISCUSSION

Secondary metabolites including alkaloids are usually synthesized by plant cells in low level to fulfill certain vital functions. As already known, they are produced mostly when the plant is exposed to stressing conditions, biotic or abiotic, such as nutrition deficiency, drought, and pathogen infection. In general, they appeared when the growth starts to go slower. Sumaryono and Riyadi (2005) demonstrated that cells of *C. ledgeriana* reached maximum growth six weeks after the last subculture and it was subsequently decreasing. For that reason we tried to apply PBZ and ABA also at the fifth week.

PBZ and ABA supplied five weeks later did not inhibit cell growth, and the alkaloids yield was not improved neither compared to those resulted from the treatments applied from the starting point. It indicates that stress condition must be present over a certain period of time to exert the effects.

When the growth curves (Figure 1a,b,c) are compared to quinoline content (Table 1), the results are relatively consistent to the rule, that the slowing down growth rate of the cells would promote the production of secondary metabolites. It was indicated by the cells treated with Tryp 02 and PBZ 5 which gave the highest quinoline content. Tryptophan is an amino acid from which quinoline alkaloids are derived (Facchini 2001). However, Robins *et al.* (1987) reported that 2 mM L-tryptophan presented some toxic effects on cell culture of *C. pubescens* that caused the cells death eventually. Furthermore, none of the quinoline alkaloids of cinchona, which were present in untreated cells, could be detected after L-tryptophan provision. In this research, tryptophan was used at 0.2 and 2 mg/l which are equivalent to 1 and 10 µM, respectively. With these levels of tryptophan, *C. ledgeriana* steadily grew even until the sixth week despite the lower rate from the untreated one; and the precursor tryptophan 0.2 mg/l enhanced the production of quinoline, particularly cinchonidine.

Cinchona bark contains 6 to 10% of total quinoline (Peter *et al.* 2007). The bark of wild species may yield quinine as high as 7%, whereas cultivated crops yields up to 15% (Barrett 1928). Peter *et al.* (2007) added that the major alkaloid in *C. ledgeriana* is quinine; cinchonidine reached the highest (3.7 to 4.9%) in the bark of *C. kartamanalis* at over 12 years of age and also high (2.1 to 2.2%) in the bark of *C. hybrida* after 6 to 8 years of age. Generally, quinoline level in cell culture is lower than that in the bark. In this work, six week-old cultures treated with tryptophan 0.2 mg/l were capable to promote the production of total quinoline to 2.8% (dry weight), which was predominated by cinchonidine. It represented 43 fold higher than in the untreated cells. After seven week of

culture, PBZ 5 mg/l produced 0.95% total quinoline, representing 16 times higher than that from the untreated cells; 97% of which was detected in the form of both quinine and quinidine.

The content of quinoline in the cell suspension cultures, especially quinine, was still very low compared to that in the bark of cinchona, but when the time span to produce quinoline through cell suspension culture is considered, 6 to 7 weeks rather than 6 to 12 years from the bark, improvements of cell suspension techniques to produce these substances is quite prospective.

It was noticed that some substances increased from the sixth to the seventh week of culture, but the others were reduced. The four quinoline alkaloids are similar in molecular structure. Quinine and cinchonine are isomers to quinidine and cinchonidine respectively. Quinine is similar to cinchonidine that the former has H and the latter has OCH at the same position; it's true as well between cinchonine and quinidine (Tadeusz 2007). This might explain that the increase and decrease of each of those four alkaloids is due to easy trans-formation among them. In addition, Robins *et al.* (1987) suggested that along with alkaloids synthesis, degradation process occurred at the same time in living cells.

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