Indonesian Journal of Science & Technology

Journal homepage: http://ejournal.upi.edu/index.php/ijost/

Alkaloids Production and Cell Growth of *Cinchona ledgeriana* Moens: Effects of Fungal Filtrate and Methyl Jasmonate Elicitors

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ABSTRACT

Cinchona alkaloids are known as antimalaria and anti-arrhythmic. Due to the long waiting time to harvest, cell culture technology is a challenge. This study aimed to determine the effects of elicitors, filtrate of two strains of endophytic fungi and methyl jasmonate (MeJA), in cell suspension culture of *Cinchona ledgeriana* on quinine and quinidine production. The cells were cultured for seven weeks in woody plant (WP) media treated with either of those elicitors in various concentrations. The cells growth was observed and the alkaloids were analyzed by HPLC. Cells treated with MeJA failed to grow that led to the cell biomass insufficiency for alkaloids determination. It indicates that the cells are quite sensitive to even low concentration of MeJA that hampered the growth. Cells treated with the filtrate of *Diaporthe* sp. M13-Millipore filtered (S2M) gave the least cell biomass but presented the highest content of both alkaloids. *Diaporthe* sp. strain M-13 is stronger as elicitor than M-23 for this plant species. Filtrate of non-virulent fungi can elevate the biosynthesis of alkaloids. This reconfirms that cultured cells are capable to produce secondary metabolites and the productivity can be increased by using an appropriate elicitor.

ARTICLE INFO

Article History:
Submitted/Received 26 Aug 2019
First revised 26 Nov 2019
Accepted 13 Jan 2021
First available online 20 Jan 2021
Publication date 01 Apr 2021

Keywords:
Cell culture,
Endophytic fungi,
Quinine
quinidine.

1. INTRODUCTION

Cinchona spp. contains secondary metabolites, including alkaloids. Cinchona bark produces 12-13% alkaloids, 70-90% of which is quinine and 1% quinidine, and the rest are other alkaloids. As the most abundant alkaloid in cinchona plants, quinine is used as an antimalarial and antipyretic medication, as a bitter flavoring in soft drinks and as a cosmetic ingredient. Quinidine is known as antiarrhythmic, anti-depressant, epilepsy therapy drugs, and used in dementia treatment (Fox *et al*., 2017). Quinine and quinidine are usually harvested directly from the bark of the cinchona plant of 7-12 years old (McCalley, 2002). The harvesting time, the long recovery period, and the re-ducing population of cinchona plants each year due to land-use shifting to cash crops have led to a vast importation of the bark flakes by our country (Indonesia). Eighty percent of the existing capacity of the cin-chona processing industry is coming from some African countries. One alternative to overcome the problem of cinchona bark scarcity is by producing quinoline alkaloids through cell culture (Ratnadewi & Sumaryono, 2010).

Cell culture is a method of producing secondary metabolites in a much shorter time than conventional methods, i.e., within weeks. Plant cells are grown in liquid medium supplemented with appropriate growth regulators and controllable environmental conditions. Through cell culture, the production of secondary metabolites can be increased by providing a certain dose of elicitor (Ratnadewi *et al*., 2013; Ncube & Staden, 2015).

Methyl jasmonate (MeJA) as an elicitor has been widely used, one of which was in *Rubia cortifolia*, to promote more purpurin biosynthesis (Koblitz *et al*., 1983). The use of endophytic fungi can also increase the accumulation of several alkaloids, such as catharanthine in *Catharanthus roseus* cell culture by *Pythium aphanidermatum* (Pasquali *et al*., 1992) and taxol in *Taxus chinensis* cell culture by *Aspergillus niger* (Wang *et al*., 2001). In cinchona cells, MeJA or any endophytic fungi have never been applied. We used microbes originated from the cinchona plant, which might interfere in the biosynthesis of the alkaloids under the natural environment. Tryptophan (Trp) is one of the precursors in quinoline biosynthesis, including quinine and quinidine, Trp at 8 mmol L^{-1} has been able to increase the synthesis of alkaloids up to six times higher compared to the control in cell culture of *Cinchona ledgeriana* (Koblitz *et al*., 1983).

Therefore, this study aimed to improve the production of quinine and quinidine in *Cinchona ledgeriana* cells by adding elicitors MeJA and its combination with Trp or filtrate of two strains of endophytic fungi (*Diaporthe* sp. strains M-13 and M-23) originated from cinchona plant, while the effects of the elicitors on the cell growth were also examined. This research result is expected to contribute to the development of cell culture technology for the production of alkaloids quinine and quinidine in a shorter time, independently of the land-use and environmental problems.

2. METHODS AND MATERIALS

2.1 Preparation of cell suspension culture and treatments

The plant material used was two weeks old *Cinchona ledgeriana* callus from the last subculture. The friable callus had been generated previously from young leaf lamina and was subcultured into basic liquid WP (woody plant) media, agitated for two weeks, and furtherly prepared for the application of the treatments according to Pratiwi *et al*. (2018). One spatula of cells (0.5-0.7 g) was put into a 120 mL flask containing 30 mL of basic WP liquid media , except that the BAP was reduced to 0 . 1 µ M , and was supplemented with an elicitor. The elicitors given were either 10% fungal crude filtrate or 1 and 5

DOI:https://doi.org/10.17509/ijost.v6i1.31479 |
p- ISSN 2528-1410 e- ISSN 2527-8045 |

mg/L MeJA. The treatments applied are summarized in **Table 1.**

Diaporthe sp. strains M-13 and M-23 are endophytic fungi isolated from *Cinchona calisaya*, the collection of IPB Culture Collection. The fungal filtrate was prepared from 7 weeks old culture on PDA media. About 5 cm^2 of culture media was taken out and mashed in 30 mL distilled water; the crushed aqueous material was passed through a filter paper. The filtrate was used to treat cell suspension cultures. The treatment media was prepared in two ways, *i.e.,* by autoclaving the whole mixture of treated media or by filtering the filtrate through a 0.45 µm Millipore filter, then incorporated the sterile filtrate into the media having been autoclaved. The culture media was adjusted to pH 5.7 before autoclaving. Media sterilization is an important process to avoid cell culture failure due to contamination with microorganisms (Abdurrahman *et al.*, 2019).

The culture was then maintained for seven weeks on a horizontal rotary shaker at 90 rpm. The room temperature was set at 26 ± 1 °C under a light intensity of 20 µmol photon/m/s for 12 hours per day. Each treatment has 15 culture flasks as replication.

2.2 Measurement of cell growth

Cell growth is represented by the volume of cell suspension culture using a non-destructive method, namely cell volume after sedimentation (CVS), according to Blom *et al.* (1992). Measurements were made once a week for seven weeks . Then the cells were harvested and weighed in fresh and oven - dried conditions.

2.3 Cell viability and size assessments

The method used to assess cell viability was 2,3,5-triphenyl tetrazolium chloride (TTC), referring to Towill and Mazur (2011). Cell viability test was carried out in the fourth and seventh weeks of culture age . Each treatment was replicated three times.

The percentage of cell viability was calculated using the formula:

$$\text{Cell viability (\%)} = \frac{\text{absorbance at 490 nm}}{\text{mg cells fresh weight}} \times 100$$

To observe the cells' condition and size, we applied 0.5% fluorescein diacetate (FDA), according to Widholm (1972). The mixture of cells and FDA solution were left for 2-5 min before being observed under a fluorescence microscope at 400 x magnification , without using fluorescence light . The FDA will cause viable cells to fluoresce under a microscope due to the FDA 's hydrolysis process becoming fluorescein in the cytoplasm (Steward *et al.*, 1999). The cell size was examined in the seventh week , with three replications . Each replication was observed in five image fields . Cell length and width were assessed using Image Raster software , and the dimension was categorized according to Vissenberg *et al.* (2001).

Table 1. Elicitor treatments in *Cinchona ledgeriana* cell suspension culture

Treatment Code	Elicitor
C	Control (no elicitor)
S1A	10% filtrate *Diaporthe* sp. - M23, autoclaved
S2A	10% filtrate *Diaporthe* sp. - M13, autoclaved
S1M	10% filtrate *Diaporthe* sp. - M23, Millipore filtered
S2M	10% filtrate *Diaporthe* sp. - M13, Millipore filtered
M1U	1 mg/L MeJA
M5U	5 mg/L MeJA
M1T	1 mg/L MeJA + 2 mg/LTrp
M5T	5 mg/L MeJA + 2 mg/L Trp

2.4 Analysis of quinine and quinidine

Analysis of quinine and quinidine was carried out using HPLC. As much as 0.1 g of oven-dried cells were extracted by grinding in a mortar with 0.3 g of $Ca(OH)_2$, then the mixture was added with 3 mL of 5% NaOH and was allowed to stand for 30 min. The results of scouring were put into Soxhlet thimble; the mortar was cleaned up from the rest of the material with methanol. Toluene, about 75 mL, was used as the main extraction solvent. The sample was extracted in Soxhlet for seven hours. Then, 100 µL of the extract was evaporated by flowing over nitrogen gas at room temperature at 0.5 - 1 mL of the mobile phase. The precipitated material was then dissolved in 2 mL of warm distilled water. Extract aliquot of 20 µL was injected into the HPLC column at 30 °C. The flow rate was 1 mL/min, with phosphate buffer as eluent. Phosphate eluent was prepared from 6.805 g of KH_2PO_4, which was dissolved in 425 mL of distilled water and adjusted to pH 3 with phosphoric acid, then mixed with 75 mL acetonitrile. The type of column used was Vp ODS C-8, 250 mm length. Quinine and quinidine were used as standards. The chromatogram was detected through a UV detector at 250 nm wavelength.

2.5 Data analysis

The experiment was designed in completely random, and the data were analyzed by ANOVA using SPSS 16. The differences among the treatment means were proceeded to further evaluation with Duncan's multiple range test at 5% of significance.

3. RESULTS AND DISCUSSION

3.1 RESULTS

3.1.1 Cell growth

Table 2 shows that control (C) cells grew the best both in the fourth and the seventh weeks of culture, while the second rank was presented by cell suspension treated with Millipore-filtered filtrate of *Diaporthe* sp. M-23 (S1M). Cells treated with fungal filtrates on average grew better than cells with MeJA treatments, although their growth was still less than the control cells **Figure 1**. Cells treated with MeJA with or without Trp had almost no growth, except in M1T (MeJA 1 mg/L+ Trp 2 mg/L) with very slow growth.

Table 2. Cell volume and viability of *Cinchona* cells in the fourth and seventh weeks of culture ages

Treatment	4th week		7th week	
	Cell Volume (mL)	Viability (%)	Cell Volume (mL)	Viability (%)
C	3.64^{d}	75.33^{f}	4.48^{d}	26.67^{c}
S1A	1.57^{b}	24.96^{d}	1.21^{ab}	4.45^{a}
S2A	1.37^{ab}	23.45^{d}	1.32^{ab}	8.75^{ab}
S1M	2.34^{c}	32.04^{e}	3.33^{cd}	22.30^{bc}
S2M	1.70^{b}	26.54^{d}	1.32^{ab}	3.13^{a}
M1U	0.81^{a}	0.71^{a}	0.97^{a}	12.56^{abc}
M5U	0.76^{a}	6.00^{b}	0.80^{a}	19.57^{abc}
M1T	0.85^{a}	15.13^{c}	2.41^{bc}	19.79^{abc}
M5T	0.85^{a}	0.68^{a}	0.94^{a}	5.60^{a}

Values sharing the same letter within the column are not significantly different at 5% level of significance.

DOI:https://doi.org/10.17509/ijost.v6i1.31479 |
p- ISSN 2528-1410 e- ISSN 2527-8045 |

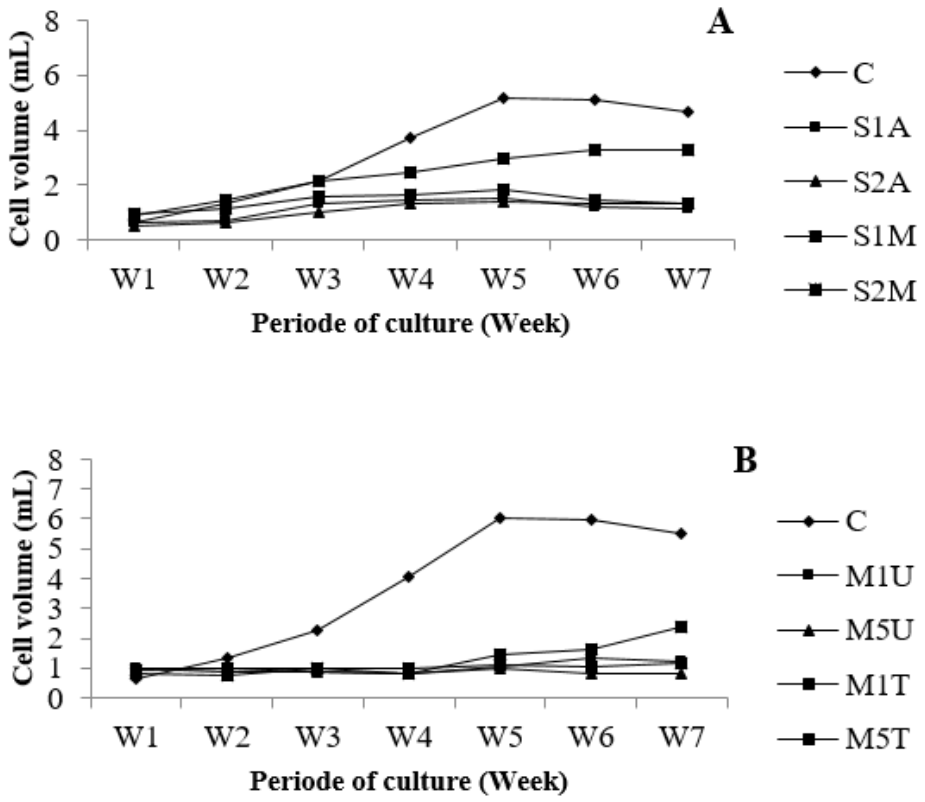

Figure 1. Growth of *Cinchona ledgeriana* cell suspension cultures treated with fungal filtrate (A) and MeJA (B).

3.1.2 Cell viability and cell size

TTC test **Table 2** explains that the highest viability in the fourth and seventh weeks was in the control cell (C). The cell viabilities of C and all the fungal filtrate treatments decreased from the fourth week to the seventh week. Meanwhile, the viability of cells with MeJA treatments increased. M1T gave the highest viability value among the cells with MeJA treatments, 15.13% in the fourth week, and 19.79% in the seventh week. **Figure 2** and **Table 3** show that the size of the control cells was more significant than those of all MeJA treated cells. Cells are categorized in three shapes, *i.e.,* long, oval, and round, based on the length and width (dimension) of each cell. In general, the cell sizes were smaller when they were treated with MeJA, and this was more obvious in long-typed cells.

3.1.3 Quinine and quinidine contents in cells suspension culture

Quinine and quinidine were analyzed from the treatments S1A, S2A, S1M, S2M, and Control (C). Cells with MeJA treatments with or without Trp in the seventh week were insufficient for extraction. The results **Table 4** show that all treated cells (S1A, S2A, S1M, S2M), including C, produced quinine and quinidine. The highest contents of quinine and quinidine were obtained in cells treated with S2M (*Diaporthe* sp. M-13 filtrate-Millipore filtered), although its quinine content was not significantly different from the other treatments.

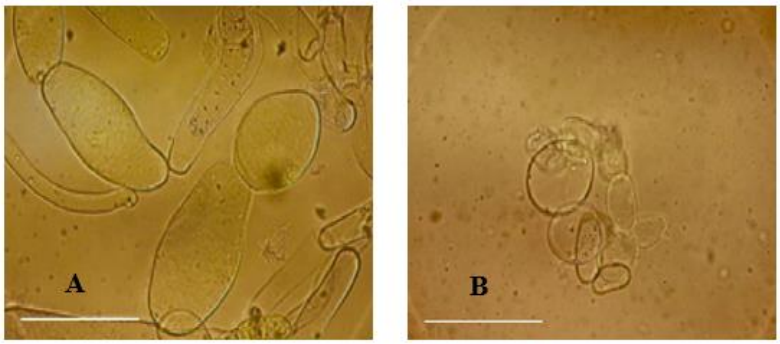

Figure 2. The performance of control cells (A) and M1T cells (B). Scale line ≈ 100 µm.

Table 3. The size of *Cinchona* cells in the seventh week of MeJA treatments

Treatment	Long Cell		Oval Cell		Round Cell	
	Length (µm)	Width (µm)	Length (µm)	Width (µm)	Length (µm)	Width (µm)
C	155.94^{b}	24.38^{b}	46.44^{a}	28.11^{a}	33.31^{b}	29.82^{ab}
M1U	102.32^{a}	19.45^{ab}	32.95^{a}	24.28^{a}	33.28^{b}	31.67^{b}
M5U	113.07^{ab}	15.97^{a}	36.97^{a}	23.59^{a}	18.49^{a}	16.79^{a}
M1T	91.12^{a}	17.71^{ab}	35.73^{a}	23.85^{a}	23.01^{ab}	20.62^{ab}
M5T	90.69^{a}	23.84^{b}	36.95^{a}	24.67^{a}	27.67^{ab}	25.91^{ab}

Values sharing the same letter within the column are not significantly different at 5% level of significance

Table 4. Quinine and quinidine in *Cinchona* cells in the seventh week

Treatment	Cell dry weight/ culture flask (g)	Content in cells (µg/g)	
		Quinine	Quinidine
C	0.230^{b}	6273.87^{a}	27817.80^{ab}
S1A	0.040^{a}	6043.07^{a}	26311.73^{a}
S2A	0.033^{a}	6289.80^{a}	27986.87^{ab}
S1M	0.153^{b}	6091.07^{a}	28875.08^{ab}
S2M	0.030^{a}	6649.60^{a}	29894.13^{b}

Values sharing the same letter within the column are not significantly different at 5% level of significance.
*The culture flask contains 30 mL of media

3.2 DISCUSSION

Control cells (C) exhibited the highest growth compared to the others. It indicates that the elicitors provided do inhibit the growth of the cells. Meanwhile, among the treated cells in general, cells with fungal filtrate grew better than cells in MeJA treatments. Cell cultures elicitated with MeJA, with or without Trp, had almost no growth. The purpose of providing Trp in this study was to stimulate the growth of cinchona cells in suspension culture, to alleviate the strong effects of MeJA. It has been known that Trp is a precursor in auxin biosynthesis, where auxin plays a vital role in plant cell growth and division. The application of Trp into culture media was able to support the growth of *Cinchona ledgeriana* cells (Ratnadewi & Sumaryono,

DOI: https://doi.org/10.17509/ijost.v6i1.31479 |
p- ISSN 2528-1410 e- ISSN 2527-8045 |

2010). Cells in M1T grew better than those in the other MeJA treatments. It was also manifested by the values of cell viability, both in the fourth and seventh weeks of culture age. In M5U and M5T, which contained 5 mg/L MeJA (equivalent to 22.3 µM), the cell growth was heavily suppressed. Elicitor MeJA is commonly employed to induce the production of secondary metabolites, *i.e.,* at the concentrations of 100 µM for taxane from *Taxus baccata* cell suspension cultures (Laskaris *et al.,* 1999) and 200 µM for taxoids in cell suspension cultures of *Taxus cuspidata* (Ketchum *et al.,* 2003). It suggested that the cinchona cell is susceptible to MeJA even at a much lower concentration. Trp seems to be more supportive when it was combined with a very low concentration of MeJA (1 mg/L). MeJA is a plant hormone that involves in defense response, and it will be generated when a wound occurs in plants. Subsequently, MeJA will result in stunted growth by inhibiting cell division (Zhang & Turner, 2008). MeJA also inhibited the growth of *Arabidopsis thaliana* leaves by reducing the cell number and size (Noir *et al*., 2013).

TTC tests on C and fungal filtrate treated cells expressed that cell viabilities in the fourth week were higher than in the seventh week. High cell growth and viability reflect the mitochondrial activity in those cells. Lower cell viability is caused by the decrease in cell metabolism along the time course, due to aging and stress experienced by the cells. However, MeJA treated cells show that the viability in the seventh week tends to increase even comparable to that of control cells, except in M5T. It leads to the notion that cells might need a longer time to adapt to MeJA, and the seventh week might only be the beginning of their revitalization.

Concerning cell size, the difference in the size of long-shaped cells is more remarkable than those of oval and round cells, between C and MeJA treated cells. Pratiwi *et al.* (2018) reported that long -shaped cells predominate in older culture , while the round cells constitute the most significant proportion in young culture , and the cell shape composition will change along with the culture age . Cell growth starts from round- shaped cell to oval and then becomes a long- shaped cell. The inhibiting effect of MeJA accumulates in older cells that gives impacts on cell size and cell suspension volume ultimately . The decrease in size is also experienced by *Arabidopsis thaliana* treated with MeJA (Zhang & Turner , 2008 ; Noir *et al*., 2013).

The results of cell extraction and HPLC analysis show that all fungal filtrate treatments (S1A, S2A, S1M, and S2M) and also C, produced quinine and quinidine alkaloids. Their contents are comparable one to another. The M-13 fungal filtrate (in S2A and S2M) is a stronger elicitor, indicated by its ability to give higher stress that effected on the little dry weight of cell biomass while inducing higher quinine and quinidine contents in the cells, compared to the M-23 fungal filtrate (in S1A and S1M). Cinchona cells without any treatment (C) are also capable of producing high levels of both alkaloids. *Diaporthe* sp. strains M-23 and M-13 are non-pathogenic endophytic fungi, isolated from *C. calisaya.* Several reports revealed that endophytic fungi are able to produce secondary metabolites originally synthesized by their host plants (Strobel *et al*., 1997 ; Nicoletti & Fiorentino , 2015). Maehara *et al. (*2001) and Radiastuti *et al. (* 2015) have proven that isolated endophytic *Diaporthe* sp . can synthesize quinine and cinchonidine.

This research employed a non-living material, which was the substances extracted from the fungi. The facts that the fungal filtrate can enhance the biosynthesis of those alkaloids prove that it contains some substances capable of activating chemical defense in plant cells, *i.e.,* the alkaloids.

Generally, quinine content is higher than quinidine in cinchona plant species. Some studies have found that quinine represents almost 80% of their total quinoline alkaloids (Hamill *et al.,* 1989; Maehara *et al.,* 2012). In cell culture, the results of this study point out that the dominant compound is quinidine, which is four times higher than quinine. Quinidine can sometimes predominate the entire quinoline alkaloids, for example, in callus (Scragg *et al.,* 1986) and root hair culture (Geerlings *et al.,* 1999) of *C. ledgeriana.*

The extraction technique used in this research resulted in much higher concentrations of quinine and quinidine than previously obtained by Ratnadewi & Sumaryono (2010), Ratnadewi *et al*. (2013), and Pratiwi *et al. (*2018). The extraction of alkaloids is more effective in a light base condition . In this case , the use of Ca(OH)$_2$ and NaOH during the grinding of dried cells, and toluene as the primary extraction solvent resulted in a higher yield of those alkaloids . McCalley (1990) and Michael (2001) have also reconfirmed that toluene is a suitable solvent for quinoline alkaloids extraction.

4. CONCLUSION

The administration of various elicitors in the cell suspension culture of *Cinchona ledgeriana* has different effects. Endophytic fungal filtrates and MeJA elicitors suppress cell growth and viability. Cell suspension culture treated with S2M elicitor (*Diaporthe* sp. M13-Millipore filtered) is the most promoting in quinoline alkaloids production, particularly quinidine. The average content of quinidine is four times higher than that of quinine. Crude filtrate of *Diaporthe* spp. M-13, a non-virulent fungus, can be used to enhance the biosynthesis of secondary metabolytes as an elicitor.

5. ACKNOWLEDGMENTS

We are very grateful to Directorate General of Research Empowerment and Development, The Indonesian Ministry of Research, Technology and Higher Education, for providing the research grant that allowed us to carry out this part of the research.

6. AUTHORS' NOTE

The authors declare that there is no conflict of interest regarding the publication of this article. The authors confirm that the data and the paper are free of plagiarism.

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DOI: https://doi.org/10.17509/ijost.v6i1.31479 |
p- ISSN 2528-1410 e- ISSN 2527-8045 |