

Cinchona cells performance in *in vitro* culture: quinine alkaloid production with application of different elicitors

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Abstract. Quinine is a dominant alkaloid in *Cinchona ledgeriana*. It is widely used in anti-malaria, anti-cramping, and anti-arrhythmia medication. In addition, it is commercially demanded for its bitter taste in certain soft drinks. The main source of quinine is the bark of Cinchona plant, which consists of 4-7% of the alkaloid. Land use shifting from perennial trees to cash-crops has resulted in a shortage of the plant material for quinine extraction. Plant cell culture has proven its success in producing secondary metabolites, including alkaloids. In principle, plant cells synthesize secondary metabolites when they are exposed to stress conditions. Therefore, in cell culture of *C. ledgeriana*, two stress-inducing agents i.e. abscisic acid (ABA) and paclobutrazol (PBZ) in combination with mannitol or sorbitol were applied to increase quinine yield. ABA and PBZ depressed the cells growth. Sorbitol caused more stress to cells than mannitol. Its combination with ABA 3 mgL⁻¹ or with PBZ 7 mgL⁻¹ mixed three weeks after culture, produced the highest concentration of quinine followed by mannitol with ABA 3 mgL⁻¹ or PBZ 7 mgL⁻¹ combined at the third week after culture. Cultured cells showed three different shapes: round, oval, and elongated. The elongated shape pre-dominated in the mature cells culture, while round cells pre-dominated in young cells culture. Those mature cells were found to contain alkaloids.

1. Introduction

Cinchona ledgeriana is one of Cinchona species containing high amount of quinine, ranging from 4-13% [1]. It is still used as an antimalarial because the resistance of *Plasmodium falciparum* parasites to quinine is lower than in synthetic drugs [2], and it is considered to be safer for pregnant women suffering from malaria [3]. In addition, quinine is active as an antibacterial. Growth inhibition of Gram-positive bacteria like *Staphylococcus aureus* and *Streptococcus pyogenes* as well as Gram-negative bacteria like *Pseudomonas aeruginosa*, *Escherichia coli*, *Proteus mirabilis*, and *Salmonella typhii* has been achieved by quinine applications [4]. Quinine has also been utilized to treat leg or



muscle cramps [5, 6] and used for anti-arrhythmic purposes. In the soft drink industry, quinine is employed as a tonic and bitter substance [7, 8].

The increasing demand for quinine, both in the food and drug industries, is still insufficiently satisfied through cinchona bark harvesting. Moreover, quinine is commonly extracted from the bark after the tree becomes at least 7 years old and the highest alkaloids content can be obtained after 20 years [1]. Many studies have been done with various plant cells by regulating alkaloid production through different manipulations to cell suspension cultures [9, 10, 11]. For quinine, the results have not yet been satisfying [9, 12, 13]. The application of elicitors as stress-inducing agents has been suggested to enhance secondary metabolites biosynthesis in plant cells [14, 15]. This research was aimed at revealing the effects of double elicitors on the growth and quinine content in cultured cells of *C. ledgeriana* while determining cell viability and cell shape. They would be further beneficial in cell selection in order to get cell lines of higher quinine production potential.

2. Materials and methods

2.1. Callus initiation

Primary callus was induced from fresh leaves of *Cinchona ledgeriana*. Young leaves were surface-sterilized with 0.4% benlate for 30 min, followed by 20% commercial NaClO for 20 min, then rinsed thrice with sterile distilled water. Their laminas were used as explants for callus production by placing them on semi-solid Woody Plant (WP) medium [16]. This medium was enriched with 15 μM picloram, 2 μM benzyladenine (BA), 1 μM phloroglucinol, 30 gL^{-1} sucrose (standard level), and was solidified with 3.5 gL^{-1} gelrite. The pH of medium was adjusted to 5.7. Explants were incubated at 25 °C in the dark for 8 weeks.

2.2. Cell suspension cultures

Fast-growing callus was transferred to baffled flasks containing WP liquid medium and homogenized for 2 weeks on a horizontal shaker, before being used for suspension culture. The liquid medium had the same formula as the callus initiation medium, except that BA was reduced to 0.5 μM [17]. Furthermore, about 0.5 g of cells were put into each Erlenmeyer flask containing 20 mL of liquid medium with various treatments. The medium composition was the same as for cell homogenization, with the addition of a growth inhibitor abscisic acid (ABA) or a growth retardant paclobutrazol (PBZ) combined with sucrose and mannitol or sorbitol. Each treatment consisted of 12 replications. The treatments were listed in Table 1.

Table 1. Treatments applied in the research and composition.

Treatment Code	Treatment composition				
	ABA (mgL^{-1})	PBZ (mgL^{-1})	Sucrose (gL^{-1})	Mannitol (gL^{-1})	Sorbitol (gL^{-1})
A1K	1		30		
A1M	1		20	5.3	
A1S	1		20		5.3
A3K	3		30		
A3M	3		20	5.3	
A3S	3		20		5.3
P7M		7	20	5.3	
P7-3M		7 (at 3rd week)	20	5.3	
P7-3S		7 (at 3rd week)	20		5.3
C (Control)			30		

The molarity of 5.3 gL⁻¹ mannitol or sorbitol is equivalent to that of 10 gL⁻¹ sucrose. The cell suspension cultures were maintained for 7 weeks on a horizontal shaker, at 80 rpm, 26 ± 1°C, under light intensity of 30 μmol photon/m²/second for 14 hours per day.

Cell growth was measured once a week, until the time of cell harvest. Cell volume after sedimentation (CVS) was used to represent cell growth, according to [18]. Cell weight was measured only at the harvest time; each treatment was represented by 5 flasks. Cell viability was also measured on the seventh week-old culture by using 2, 3, 5-triphenyl tetrazolium chloride (TTC), according to [19] with slight modification. A total of 50-100 mg of fresh cells were put into a test tube and 3 mL of 0.6% TTC solution in distilled water were added. The cells were left overnight at 25 °C in the dark room. Then 7 mL of 96% alcohol were added into the tube, and incubated for 7 minutes in a water bath at 60 °C. The cells were centrifuged twice at 2000 rpm, for 6 minutes at a time. Absorbance was determined at 490 nm on the supernatant using a spectrophotometer. Percentage of cell viability was calculated by the formula:

$$\text{Cell viability} = \frac{\text{Absorbance at 490 nm}}{\text{mg fresh cells}} \times 100\%$$

2.3. Quinine analysis

Quinine analysis was performed using UFLC (Ultra-Fast Liquid Chromatography; Shimadzu Prominence 20 AD type). The analysis was conducted in 3 replications. Cells were analyzed in 7-week-old cultures.

An oven-dried sample (0.1 g) was extracted with 1 mL of phosphate buffer pH 7, then filtered using filter paper. This extract was processed through gel chromatography column F 254 (7 cm length; 1 cm diameter). The eluent used was a mixture of phosphate buffer pH 7 and acetonitrile (7:3). The third fraction which resulted in the first clean extract was used for quinine analysis. HPLC with ODS C-18 VP column was used with an eluent mixture of H₂O:acetonitrile: glacial acetic acid (81:18:1); column length 250 mm, temperature 30°C, and flow rate 1 mL/min. Quinine content was calculated by comparing the peak area of the sample with that presented by quinine standard. The chromatogram was scanned with a UV detector at 250 nm.

2.4. Histochemical test of alkaloids in cultured cells

The cells were harvested after 7 weeks in culture, then centrifuged at a low speed of 20 rpm for 5 minutes. The pellets were suspended with a few drops of distilled water. A small amount of cells were placed in an Eppendorf tube and some drops of Wagner reagent were applied to let the reaction go for 1 minute. The presence of alkaloid in cells was characterized by a brownish red color. As a negative control, the sample was immersed in 5% tartaric acid in 95% ethanol for 48 hours at room temperature, and then it was incubated with Wagner reagent for 1 minute [20].

2.5. Cell observation on size and shape

Cell observation was performed when the cultures aged three and seven weeks. The parameters observed were the length and width of cell to determine the shape category according to [21]. The number of round, oval, and elongated cells were counted from 5 image fields for each sample; triplicate observations were carried out. Measurement of length, width and diameter was performed using image analysis software Image Raster v 2.1.

A completely randomized design was used in this study. The collected data were analyzed in ANOVA; significant differences among the treatments were further proceeded to Duncan's Multiple Range Test (DMRT) at the 5% level of significance.

3. Results and discussion

3.1. Cell growth and viability in suspension culture

Callus initiated from leaf explants appeared on the second week. The callus was bright yellow and friable. The administration of elicitors showed its effect on cell growth. In the seventh week of culture, the highest cell growth (volume) was shown by A3K treatment, which was 11.51 mL, while the most depressed cell growth was shown by P7M, A1K, P7-3S and A3S (Table 2). Moderate growth rates were shown by cells in A1M, A3M, P7-3M, and Control. Cell growth reached its maximum in the sixth week, and simultaneously began to decrease in the seventh week (Figure 1).

Table 2. Cell volume and cell viability of *C. ledgeriana* in suspension culture at the seventh week of culture.

Treatments	Cell Volume (mL)	Cell Viability (%)
A1K	5.27 ^a	23.33 ^{bcd}
A1M	7.64 ^{cd}	24.84 ^{cd}
A1S	7.09 ^{bc}	21.38 ^{bcd}
A3K	11.51 ^e	26.09 ^{cd}
A3M	8.06 ^d	13.78 ^{ab}
A3S	5.74 ^{ab}	23.49 ^{bcd}
P7M	5.20 ^a	10.89 ^a
P7-3M	9.35 ^d	16.82 ^{abc}
P7-3S	5.73 ^a	31.25 ^{de}
C	9.69 ^d	40.85 ^e

*The numbers followed by the same letter in the same column were not significantly different based on DMRT at the 5% level of significance.

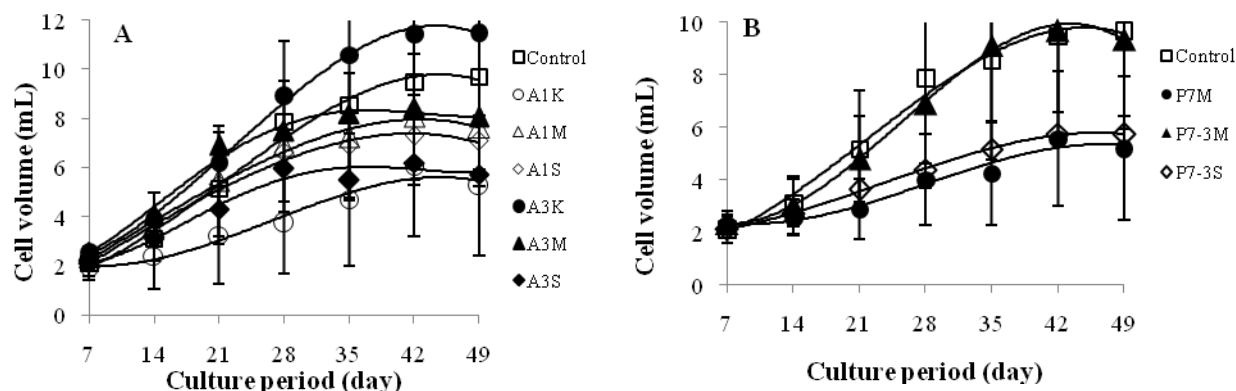


Figure 1. *Cinchona ledgeriana* cells growth in various elicitors treatment combinations.

Notes: (A) Combination of ABA and mannitol or sorbitol; (B) Combination of PBZ and mannitol or sorbitol.

Superior cell growth was observed in 3 mgL⁻¹ ABA with standard concentration of sucrose (A3K). ABA at 3 mgL⁻¹ promoted cell growth compared to 1 mgL⁻¹. PBZ exhibited more severe inhibition on cell growth, but when it was incorporated in the third week of culture, which was the beginning of the linear phase of cell growth, lighter suppression occurred whereas the inhibition process was more pronounced when PBZ was given earlier (Figure 1). When PBZ was added into the culture media in the fifth week of culture, it did not significantly reduce the cell growth [13]. Sorbitol was more disadvantageous than mannitol when it was combined with either ABA or PBZ. ABA is also known to play a complex role in plant growth, either as a promoter or inhibitor. The addition of ABA in cell culture helps cells to cope with water deficit and osmotic stress [22] that may be due to sugar content.

The use of alcohol sugar (mannitol or sorbitol) as a partial substitution of sucrose diminished energy supply to the cells since alcohol sugar is not easily metabolized, but it contributes in the medium's osmotic potential that reduced the cells capacity to absorb water from the media [23]. Consequently, cell growth in mannitol or sorbitol treatment combinations became lower. This explains why ABA increased the rate of cell growth on a medium without sugar substitution, increasing cell volume and cell weight compared with other treatments, including Control (C).

Without elicitor, Control (C) gave the highest cell viability (Table 2). The time of PBZ administration provided a different effect; PBZ added since the beginning of culture (P7M) caused lower cell viability compared with that when PBZ was added after three weeks (P7-3M and P7-3S). Cell volume represented the accumulating growth result during seven weeks of culture, while cell viability was the value of a one-time measurement at the seventh week. There seems to be inconsistency in several cases when the cell growth corresponded with cell viability. It might be due to the fact that six weeks of culture is the maximum for cells to grow, beyond which the cells rapidly decreased their metabolic activities. The decrease in metabolic activity may be caused by the cell age, and additionally the elicitors in cell cultures had created stressful conditions that had great impact on cell viability. The cells treated with elicitors showed a decrease in growth rate in the seventh week, while Control cells still showed a slight increase.

3.2. Quinine content

Various combinations of elicitors in cell culture were employed in order to obtain an optimal yield of quinine, higher than those previously gained. Quantitative analysis of quinine with HPLC showed that treatment with elicitors generally had a marked effect on increasing quinine content than without elicitors (C) (Table 3). The highest quinine content was obtained from A3S treatment ($92.39 \mu\text{g g}^{-1}$) followed by P7-3S ($85.57 \mu\text{g g}^{-1}$) and A3M ($71.00 \mu\text{g g}^{-1}$), but the total quinine produced in each culture flask showed different results. The A3K treatment with the most abundant cell biomass provided the highest total quinine of $21.87 \mu\text{g}$ per 20 mL of medium in a flask, followed by A3S ($19.03 \mu\text{g}/20 \text{ mL}$).

Table 3. Quinine content of *C. ledgeriana* in seven-week-old cultures.

Treatments	Cell dry weight (g)	Quinine content ($\mu\text{g g}^{-1}$)	Total quinine/culture flask ¹ (μg)
A1K	0.11 ^{ab}	34.19 ^a	6.17 ^a
A1M	0.12 ^{abc}	45.23 ^{ab}	8.56 ^{ab}
A1S	0.11 ^{ab}	43.20 ^{ab}	7.63 ^a
A3K	0.21 ^d	61.99 ^{abc}	21.87 ^d
A3M	0.12 ^{abc}	71.00 ^{bcd}	14.21 ^{bc}
A3S	0.12 ^{abc}	92.39 ^d	19.03 ^{cd}
P7M	0.07 ^a	56.05 ^{ab}	6.95 ^a
P7-3M	0.14 ^{bc}	63.82 ^{abc}	14.22 ^{bc}
P7-3S	0.08 ^a	85.57 ^{cd}	11.38 ^{ab}
C	0.17 ^{cd}	33.46 ^a	9.27 ^{ab}

¹The culture flask contained 20 mL of media.

The numbers followed by the same letter in the same column were not significantly different based on DMRT at the 5% level of significance.

The use of ABA in low concentration of 1 mg L^{-1} and high concentration of 3 mg L^{-1} showed different patterns in quinine content. Treatment with 3 mg L^{-1} ABA induced quinine content more than 1 mg L^{-1} . P7M treatment had lower quinine content ($56.06 \mu\text{g g}^{-1}$), significantly different from P7-3S ($85.57 \mu\text{g g}^{-1}$), while the quinine content in P7-3M was in between. In reality, however, total quinine is more appreciable and useful; therefore, high content must be accompanied by high cell biomass. Indeed, PBZ induced high content of quinine but the biomass produced was very low due to its strong inhibition of the cell growth particularly if PBZ was provided from the beginning of the culture. P7-

3M treatment was promising since it produced sufficiently good cell biomass and quinine content which resulted in a moderate total amount of quinine (14.22 μg).

Overall, quinine content obtained in this study was smaller than those reported previously [13], which achieved the highest yield of quinine of 1206.70 $\mu\text{g g}^{-1}$, whereas in this study the highest yield was 92.39 $\mu\text{g g}^{-1}$. These results were obtained by different extraction methods and by the use of different clones of *C ledgeriana*. The plant materials used in this research were collected from an open plantation. In cell suspension cultures, the role of media composition is very important to support the yield of quinoline alkaloids, especially quinine. Some studies on *C ledgeriana*'s quinine were carried out by using various combinations of culture media. As much as 0.11 μg quinine per g cell fresh weight was obtained by combining 0.5 mg L^{-1} 2,4-D and 0.1 mg L^{-1} IBA [9]; zeatin riboside and IAA were able to increase quinine to reach 2.2 $\mu\text{g g}^{-1}$ of cell fresh weight [24]; 1 $\mu\text{g g}^{-1}$ quinine was obtained by cell suspension culture in a medium containing of 3.5 mg L^{-1} NAA and 0.1 mg L^{-1} BA [12].

Leaves of *Cinchona ledgeriana* from a one-year-old tree contained 90 $\mu\text{g g}^{-1}$ quinine [1]. Normally, quinoline alkaloids are extracted from tree bark of seven to twelve-year-old trees [25]. Samples from the Bukit Tunggul plantation, West Java, Indonesia have 3.9-6.1% quinine in *C ledgeriana* and 2.4-2.8% in *C succirubra*, extracted from 7-8 year-old trees (personal communication with Santosa TB, the Operational Director of PTPN VIII March 2018). Cell suspension culture of *C ledgeriana* possessed very low capacity in producing quinine. Several factors might be involved in the production process, such as the availability of precursors, important enzymes in the biosynthesis, and also maximal level of quinine required by individual cell to maintain its homeostasis. According to [26], quinine and quinidine are substantially more toxic to cultured cells than cinchonine and cinchonidine. In intact plants, the site of biosynthesis may be away from the site of its accumulation; a translocation is required to avoid toxic levels of a substance in an individual producing cell or tissue. Cultured cells have no such a system.

3.3. Cells size and shape

The cultured cinchona cells were characterized by three different shapes: round, oval, and elongated (Figure 2). These cells were single and clustered. Measurements in the third week showed cells with a round-shape predominated over oval- and elongated-shaped cells, while in the seventh week, cells with elongated-shape were found in larger number than oval- and round-shapes (Figure 3). Oval cells were present in moderate number either in the third week or in the seventh week. It is assumed that young cinchona cells were round-shaped, which progressively extended to oval then elongated as the age of the cell increased. *Peganum harmala* cells grown *in vitro* in the presence of 2 mg L^{-1} 2,4-D composed of round, oval and elongated cells at the exponential and early stationary phase in the cell growth curve, then elongated and large cells predominated in the exponential phase [27]. In cinchona cell cultures, the abundance of round and elongated cells was influenced by the treatment; ABA treatments which demonstrated growth promotion up to six weeks of culture provided round cells more than PBZ. It was thought that the round cells have the capacity to divide, not the long cells.

Certain cellular forms may indicate the presence of secondary metabolites, since the cell shape can change during their accumulation [23]. Previous research suggested that the process of cell expansion is a process of cell specialization to produce secondary metabolites [28]. The results obtained by [20] that quinine content in cells aged seven weeks were quantitatively much higher than in six-week-old cells in all treatments applied, indicated that elongated cinchona cells accumulate quinine alkaloid more abundantly than the round or young cells. The fact that strong elicitor combinations such as A3S, P7-3S and A3M enhanced quinine biosynthesis that led to higher concentration in quinine obtained, suggested that low growth rate of cells resulted in those treated media had more elongated cells. In *Peganum harmala* cultures where elongated cells predominated, they accumulated large amounts of β -carboline and serotonin alkaloids in their vacuoles [27]. Similarly, in strawberry cell suspension culture, elongated cells have higher anthocyanin level than round cells [29].

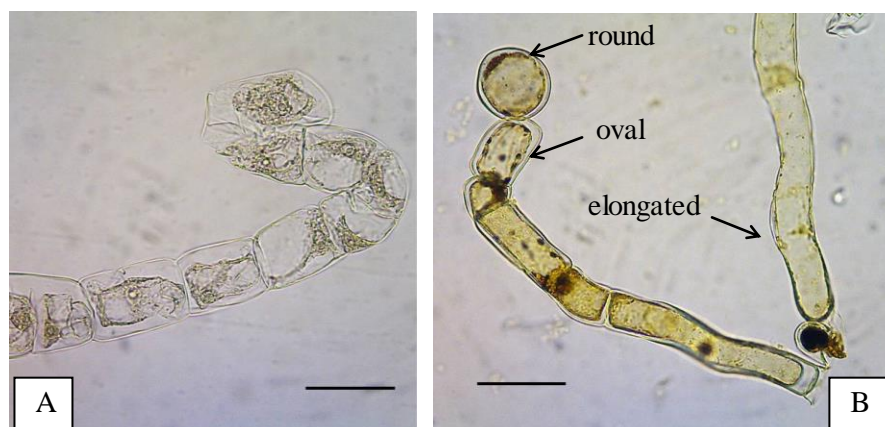


Figure 2. Cinchona cell shapes. Negative control (A), positive result (B) of histochemical test on various cell shapes accumulating alkaloids. Bar = 100 μm .

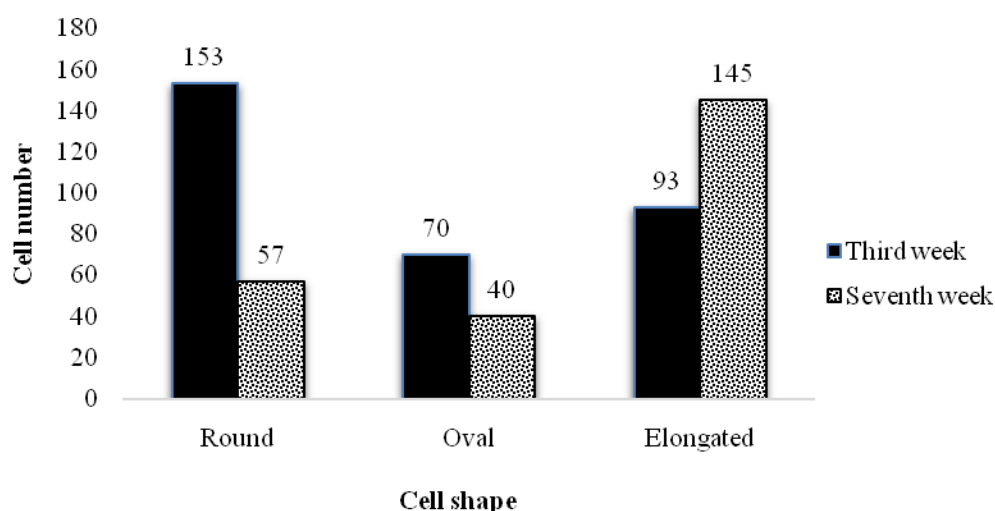


Figure 3. Distribution of cell shape variations in *C ledgeriana* suspension culture at the third and seventh week of culture.

4. Conclusion

Double elicitors in *C ledgeriana* cell culture inhibited cell growth but promoted higher concentration in quinine alkaloids. The best combination of elicitors for quinine content was 3 mgL^{-1} ABA with sorbitol as a partial substitution of sucrose. Sorbitol gave a higher abiotic stressing effect than mannitol. The large cells biomass and quinine content determined the total quinine yield; the highest was obtained from 3 mgL^{-1} ABA without sugar substitution. The characteristic of cinchona cell shape was round, oval, and elongated. All cell types were detected containing alkaloids, but cultures with predominant elongated cells contained quinine more intensively at the seventh week. In addition, round cells were widely found in ABA treatments, indicating active cell division that extrapolated through the cell growth rate.

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References

- [1] Staba E J 1988 *Alkaloid production from Cinchona cell and organ systems Genetic Manipulation of Woody Plants* eds J W Hanover, D E Keathley, C M Wilson, G Kuny (New York: Plenum Pr) pp 313-328
- [2] Zalis M G, Pang L, Silveira M S, Milhous W K and Wirth D F 1998 Characterization of *Plasmodium falciparum* isolated from the Amazon region of Brazil: evidence for quinine resistance *AJTMH* **58** 630
- [3] Achan J, Talisuna A O, Erhart A, Yeka A, Tibenderana J K, Baliraine F N, Rosenthal P J and D'Alessandro U 2011 Quinine: an old anti-malarial drug in modern world: role in the treatment of malaria *Malaria J.* **10** 1
- [4] Kharal S A, Hussain Q, Ali S and Fakhuruddin 2009 Quinine is bactericidal *JPM* **59** 208
- [5] Mandal A K, Abernathy T, Nelluri S N and Stitzel V 1995 Is quinine effective and safe in leg cramps? *J. Clin. Pharmacol.* **35** 588
- [6] El-Tawil S, Al Musa T, Valli H, Lunn M P T, El-Tawil T and Weber M 2010 *Quinine for Muscle Cramps* (Hoboken (US): John Wiley & Sons)
- [7] Anderson L A, Philipson J D and Robert M F S 1986 *Aspect of alkaloid production by plant cells cultures* (Cambridge: Cambridge University Pr)
- [8] Sheldon R, Duff H and Koshman M L 1995 Antiarrhythmic activity of quinine in humans *Circulation* **92** 2944
- [9] Robins R J, Payne J and Rhodes M J C 1986 Cell suspension cultures of *Cinchona ledgeriana*; I. growth and quinoline alkaloid production *Planta Med.* **52** 220
- [10] El-Sayed M and Verpoorte R 2002 Effect of phytohormones on growth and alkaloid accumulation by a *Catharanthus roseus* cell suspension cultures fed with alkaloid precursors tryptamine and loganin *Plant Cell, Tiss. Organ Cult.* **68** 265
- [11] Narasimhan S and Nair G M 2004 Effect of auxins on berberine biosynthesis in cell suspension culture of *Coscinium fenestratum* (Gaertn.) Colebr-a critically endangered medicinal liana of Western Ghats *Indian J. Exp. Biol.* **42** 616-9
- [12] Staba E J and Chung C T A 1987 Effects of age and growth regulators on growth and alkaloids production in *Cinchona ledgeriana* leaf-shoot organ cultures *Planta Med.* **53** 206
- [13] Ratnadewi D and Sumaryono 2010 Quinoline alkaloids in suspension cultures of *Cinchona ledgeriana* treated with various substances *Hayati J. Biosci.* **17** 179
- [14] Huang C, Qian Z G and Zhong J J 2013 Enhancement of ginsenoside biosynthesis in cell cultures of *Panax ginseng* by N,N'-dicyclohexylcarbodiimide elicitation *J. Biotechnol.* **165** 30
- [15] Panwar G S and Guru S K 2015 Stimulation of reserpine production in the whole plant culture of *Rauwolfia serpentina* L. by elicitors and precursor feeding *J. Biochem. Biotechnol.* **24** 49
- [16] Llyod G and McCown B 1981 Commercially feasible micropropagation of mountain laurel *Kalmia latifolia* by use of shoot tip culture *Combined Proc. Int. Plant Propagators' Soc.* **30** 421
- [17] Sumaryono and Riyadi I 2005 Pertumbuhan biak kalus dan suspensi sel tanaman kina (*Cinchona ledgeriana* Moens) *Menara Perkebun.* **73** 1
- [18] Blom T J M, Kreis W, Van Iren F and Libbenga K R 1992 A non-invasive method for the routine estimation of fresh weight in batch suspension cultures *Plant Cell Rep.* **11** 146
- [19] Benson E E 1994 *Cryopreservation Plant Cell Culture: A Practical Approach* 2th Ed eds R A Dixon, R A Gonzales (Oxford: IRL) chapter 7 pp 147-166
- [20] Furr M and Mahlberg P G 1981 Histochemical analyses of laticifers and glandular trichomes in *Cannabis sativa* *J. Nat. Products.* **44** 153
- [21] Vissenberg K, Feijo J A, Weisenseel M H and Verbelen J-P 2001 Ion fluxes, auxin and the induction of elongation growth in *Nicotiana tabacum* cells *J. Ex. Bot.* **52** 2161
- [22] Seijo G 2000 Effects of preculture with sucrose and ABA on cell suspension water status and its relation with vitrification resistance *Revista Brasileira Fisiologia Vegetal* **12** 166

- [23] Dumet D, Engelmann F, Chabrillange N, Dussert S and Duval Y 1994 Effects of various sugars and polyols on the tolerance to desiccation and freezing of oil palm polyembryonic cultures *Seed Sci. Res.* **4** 307
- [24] Rhodes M J C, Payne J and Robins R J 1986 Cell suspension cultures of *Cinchona ledgeriana*; II. the effect of a range of auxins and cytokinins on the production of quinoline alkaloids *Planta Med.* **52** 226
- [25] Kurian A, Sankar M A and Peter K V 2007 *Medicinal Plants* (New Delhi: New Delhi Publ Agency)
- [26] Walton N J, Parr A J, Robins R J and Rhodes M J 1987 Toxicity of quinoline alkaloids to cultured *Cinchona ledgeriana* cells *Plant Cell Rep.* **6** 118
- [27] Khafagi I K 2007 Generation of alkaloid-containing idioblasts during cellular morphogenesis of *Peganum harmala* L cell suspension cultures *Am. J. Plant Physiol.* **2** 17
- [28] Kim S W, Jung K H, Kwak S S and Liu J R 1994 Relationship between cell morphology and indole alkaloid production in suspension cultures of *Catharanthus roseus* *Plant Cell Rep.* **14** 23
- [29] Forni C, Frattarelli A and Damiano C 1999 Different size, shape and growth behaviour of cells in suspension cultures of strawberry (*Fragaria x ananassa* Duch) *Plant Biosystem.* **133** 205