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Cell suspension culture of Zedoary (*Curcuma zedoaria* Roscoe)

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Abstract. We report here the protocol for Zedoary (*Curcuma zedoaria* Roscoe) callus and cell suspension cultures. The MS medium supplemented with 2% sucrose, 1.0 mg/l 2,4-D and 1.0 mg/l BA was effective for callus induction from *in vitro* leaf-base explants of Zedoary. During subcultures, secondary proliferated calli were subsequently produced from initial induced calli on the MS medium with 0.5 mg/l 2.4-D and 0.5 mg/l BA. These calli were light yellow in color, compact and friable. The cell suspension culture for Zedoary was established using 3 g fresh weight inoculum in a batch culture on the MS medium supplemented with 3% sucrose, 1.5 mg/l 2,4-D and 0.5 mg/l BA. The highest biomass of 10.44 g fresh weight (0.66 g dry weight) was obtained after 14 days of culture in 50 ml liquid medium of 250 ml Erlenmeyer flask with shaking speed of 120 rpm. Results from this study might be a well established foundation for further studies on *Curcuma zedoaria* Roscoe in order to serve as a potential source for secondary metabolites production in large scale.

Keywords: Callus, cell biomass, cell suspension, *Curcuma zedoaria*, medicinal plant

1. Introduction

Zedoary (*Curcuma zedoaria* Roscoe) plant, a vegetatively propagated species of the Zingiberaceae family, is an aromatic herbaceous plant with a rhizome growing mainly in South Asian and South-East Asian countries, and China [1]. Zedoary is a valuable medicinal plant, the essential oil obtained from rhizome has been reported to have antimicrobial activity and be clinically used in the treatment of cervical cancer, the water extract of Zedoary demonstrated antimutagenic activity [2]. It has been also used for stomach diseases, hepatoprotection [3], treatment of blood stagnation, and promoting menstruation as a traditional medicine in Asia [4]. Furthermore, the Zedoary has anti-inflammatory potency related to its antioxidant effects [3].

Higher plants are a valuable source of wide range of secondary metabolites, which are used as pharmaceuticals, agrochemicals, flavours, fragrances, colours, biopesticides and food additives [5]. In the end of 1960s, plant cell culture technologies were introduced as a tool for both studying and producing plant secondary metabolites. A highly potent

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secondary metabolites that is used in pharmaceuticals and food additives have been produced through plant cell suspension cultures in large-scale [6, 7]. Cell suspension culture is a requirement for the production of chemicals from plants in a way quite similar to that used for microorganisms, where the utilization of bioreactor becomes feasible [8]. The purpose of this study is to establish an efficient suspension cell culture protocol for *C. zedoaria* as a starting point to produce bioactive compounds in plant cell culture.

2. Materials and methods

2.1. Callus culture

Leaf-base explants of 0.5×0.5 cm were excised from *in vitro* growing Zedoary plants on the Murashige and Skoog (MS) [9] solid medium supplemented with 2% (w/v) sucrose, 20% (v/v) coconut water, and 2 mg/l naphthaleneacetic acid (NAA) [10]. The explants were placed on the MS solid medium supplemented with 2% (w/v) sucrose, 0.25 to 4.0 mg/l 2,4-dichorophenoxyacetic acid (2,4-D), and 0.25 to 4.0 mg/l benzyladenine (BA) for callus induction and its proliferation. The pH of the medium was adjusted to 5.8, and then it was autoclaved at 121^{o}C for 15 min. The cultures were incubated at 25±2^{o}C under an intensity of 2,000-3,000 lux with a photoperiod of 10-h day light.

2.2. Establishment of cell suspension culture

Cell suspension cultures were initiated through the agitation of 3 g of callus in 250 ml Erlenmeyer flasks containing 50 ml MS liquid medium supplemented with 2% (w/v) sucrose, 0.5 mg/l 2,4-D and 0.5 mg/l BA for 10 days at 120 rpm until a suspension of free cells formed. Then, 3 g of cells from initial culture was transferred on the MS medium supplemented with different plant growth regulators (2,4-D and BA) and sucrose for investigation of biomass production. The suspensions were placed on a rotary shaker at 120 rpm for 18 days under the same conditions as for the callus culture except an intensity of 500 lux.

Samples were obtained every two days to determine the cell biomass in both fresh and dry weights. For measurement of fresh cell weight, the cells in the suspension culture were filtered, washed with distilled water, collected, and weighed. The dry cell weight was determined by drying the fresh cell biomass at 50^{o}C until a constant weight was attained.

Growth index = Final fresh cell weight/Initial inoculums fresh cell weight.

2.3. Statistical analysis

The experiments of callus and cell suspension culture were conducted with a minimum of three replicates. All experiments were repeated three times. The data were analyzed by mean ± standard error followed by comparison of the means with the Duncan's test at $p<0.05$.

3. Results and discussion

3.1. Callus induction and proliferation

As shown in Table 1, the effects of 2,4-D and BA on callus induction were initially tested using the leaf-base explants of *in vitro* Zedoary plant. The MS medium with 1.0 mg/l 2,4-D and 1.0 mg/l BA showed the strongest induction with 46% of explants produced callus; calli were white in color and soft (Fig 1A). The other combinations of plant growth regulators resulted in non-growth or poor growth of

Zedoary callus. No callus was formed on medium without 2,4-D and BA. At high concentrations of 2,4-D (3.0-4.0 mg/l) and BA (3.0-4.0 mg/l), or low concentration of 2,4-D (0.5 mg/l) and BA (0.5 mg/l) the callus obtained was white in color and viscous (Fig 1B). It was found that medium concentrations of 2,4-D (1.0-2.0 mg/l), in combination with BA (1.0-2.0 mg/l), had significant effects on callus formation. Several studies had been reported regarding the effects of plant growth regulators on callus growth of Zedoary [8, 11] found callus was induced from root segments of *in vitro* Zedoary plants. The explants were cultured on the MS medium supplemented with 3% sucrose, 13.4 µM NAA and 2.2 µM BAP in the dark. According to [12], no callus formation from the explants of Zedoary on the MS medium with 2,4-D. Callus induction and growth were obtained by inoculating root segments on the MS medium supplemented with 1.0 mg/l NAA and incubation in the dark. In this work, other plant growth regulators treatments (NAA and kinetin) also had been done on callus induction from Zedoary (data not shown) but were terminated due to several problems such as weak callus induction frequency (NAA) or browning callus after three times of subculture (kinetin).

Table 1. Callus induction and morphogenesis of Zedoary leaf-base explants

2,4-D (mg/l)	BA (mg/l)	% explant produced callus	Callus induction	Callus morphogenesis
0.0	0.0	0.00	-	-
0.5	0.5	15.74^{c}	++	White and viscous
1.0	1.0	46.01^{a}	++++	White and soft
2.0	2.0	40.10^{b}	++++	White and soft
3.0	3.0	10.20^{d}	+++	White and viscous
4.0	4.0	8.20^{e}	++	White and viscous

-: no induction; +: induction; ++: low production of callus; +++: medium production of callus; ++++: high production of callus
Different letters indicate significantly different means using Duncan's test ($p<0.05$)

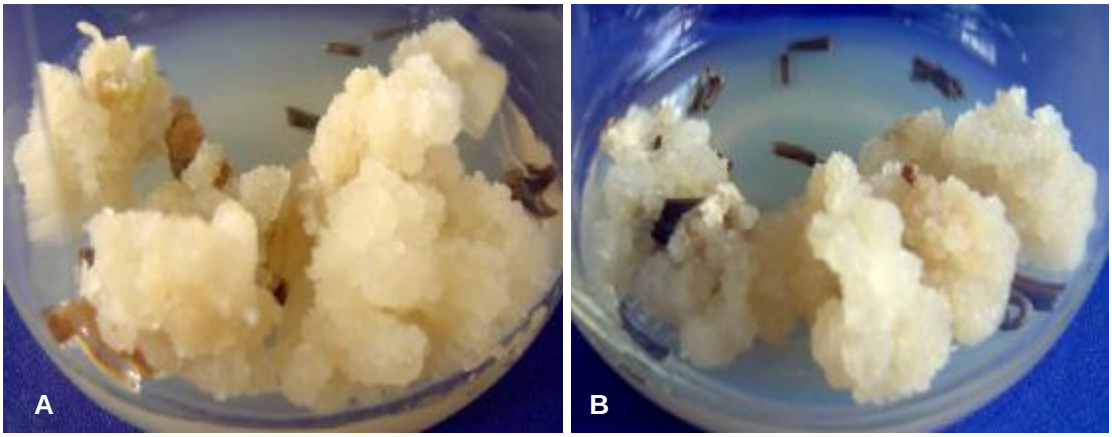

Fig. 1. Callus induction culture of Zedoary. A: White and soft callus, B: White and viscous callus.

White and soft calli (primary calli) were transferred on the MS media supplemented with different concentrations of 2,4-D and BA for proliferation. Calli obtained from the induction medium with 2.0 mg/l 2,4-D and 2.0 mg/l BA turned brown and dead in all subcultures. Calli obtained from the induction medium with 1.0 mg/l 2,4-D and 1.0 mg/l BA developed into secondary calli on the medium containing 0.5 mg/l 2,4-D and 0.5 mg/l BA (Table 2). Other concentrations of 2,4-D and BA did not show any positive response for callus proliferation in any of the tested formulars of explants when evaluation was carried out four weeks after subculture. The secondary calli which were light yellow in color, compact and friable obtained after four weeks of culture were subcultured and maintained in the fresh medium with the same composition every two weeks (Fig. 2A).

Table 2. Growth ability and morphogenesis of Zedoary primary callus

2,4-D (mg/l)	BA (mg/l)	Callus growth	Callus morphogenesis
0.25	0.25	++	White and viscous
0.50	0.50	++++	Light yellow, compact and friable
1.00	1.00	++	White and soft
2.00	2.00	++	White and viscous
4.00	4.00	-	Brown and dead

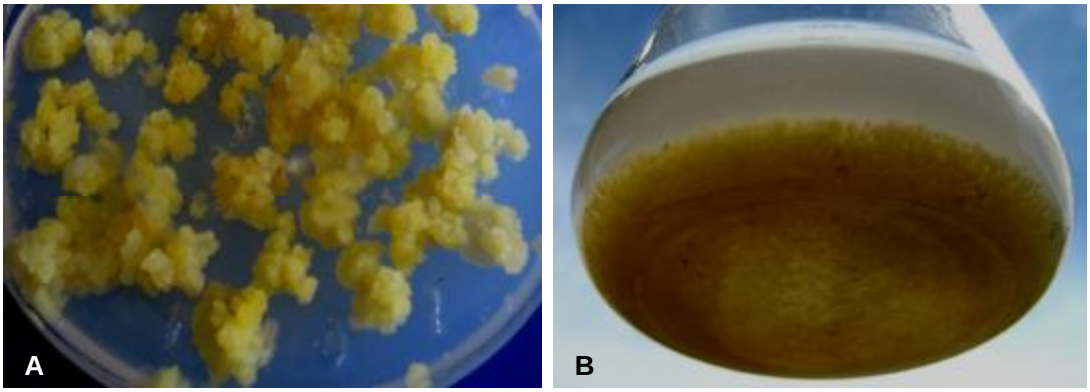

Fig. 2. Secondary callus and suspension cell of Zedoary. A: Light yellow, compact and friable callus, B: Suspension cell

3.2. Cell suspension culture for biomass production

In order to investigate cell biomass accumulation, a suspension culture was established. Approximately 3 g fresh weight of callus was transferred in 50 ml the liquid MS medium containing 0.25 to 2.5 mg/l of 2,4-D and 0.5 mg/l BA. Suspension cells were initially generated as shown in Fig. 2B within 2 weeks of culture. In order to maintain the suspension culture, 3 g of cells was transferred to fresh MS liquid medium at 10 days interval. Growth of cell was also determined by fresh and dry weight measurement (Fig 3A and 3B). The fresh and dry weights of cells were recorded every two days until 18 days of culture time. It was observed that cell biomass was increased by culture time (data not shown). The MS medium supplemented with 1.5 mg/l 2,4-D and 0.5 mg/l BA showed the highest biomass accumulation compared to other combinations of 2,4-D and BA. Day 14^{th} showed the maximum biomass accumulation with 7.22 g of fresh weight (approximately 0.55 g of dry weight) (Table 3).

Table 3. Effect of plant growth regulators on the production of Zedoary cell biomass

BA (mg/l)	2,4-D (mg/l)	Fresh weight (g)	Dry weight (g)	Growth index
0.5	0.25	5.12^{bc}	0.35^{b}	1.71
0.5	0.5	5.91^{b}	0.44^{ab}	2.05
0.5	1.0	6.20^{b}	0.46^{ab}	2.06
0.5	1.5	7.22^{a}	0.55^{a}	2.41
0.5	2.0	6.03^{b}	0.43^{ab}	2.19
0.5	2.5	6.01^{b}	0.42^{ab}	2.18

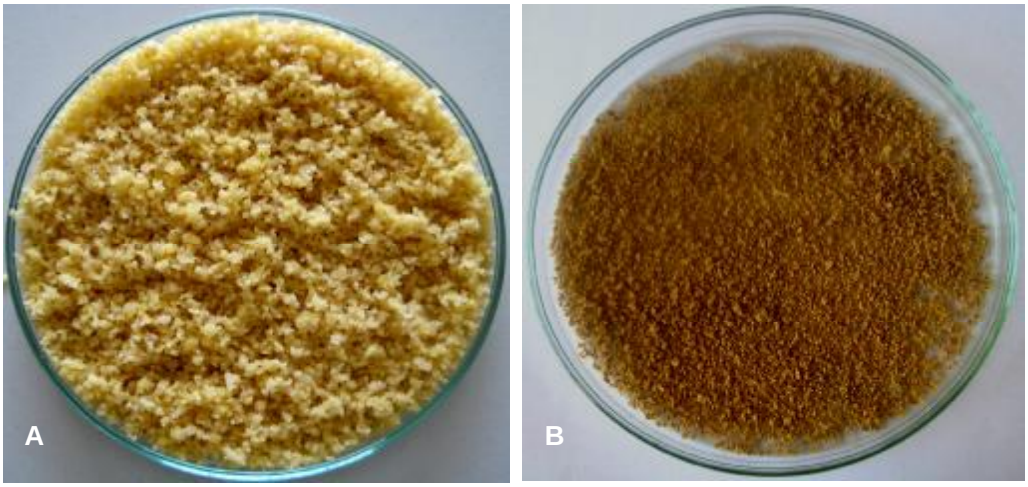

Fig. 3. Cell biomass of Zedoary. A: Fresh cell biomass, B: Dry cell biomass.

As shown in Table 4, the concentrations of sucrose to significantly affect on the biomass accumulation of cell, highest fresh cell weight were attained in media containing sucrose concentration of 3% (10.44 g with growth index of 3.48). However, despite sucrose being an indisputably important carbon and energy source, increasing its concentration from 4 to 6% resulted in fresh cell weight reductions and significantly reduced at sucrose concentration of 7%. This decline in performance might be attributed to the inhibition of nutrient uptake as the osmotic potential was enhanced and the medium became more viscous. As have shown in *Vitis vinifera* that a higher concentration of sucrose can act as an osmotic agent, with mannitol having a similar effect on growth [13]. Additionally, this retardation in growth could be caused by a cessation in the cell cycle when nutrients are limited and sucrose concentrations are higher [14, 15].

Table 4. Effect of sucrose concentration on the production of Zedoary cell biomass

Sucrose concentration (g/l)	Fresh weight (g)	Dry weight (g)	Growth index
20	7.22^{c}	0.55^{b}	2.41
30	10.44^{a}	0.66^{a}	3.48
40	8.85^{b}	0.64^{a}	2.95
50	8.80^{b}	0.65^{a}	2.93
60	8.75^{b}	0.70^{a}	2.92
70	6.75^{c}	0.60^{a}	2.25

Typical cell growth curves constructed from suspension culture are shown in Fig.4. These curves indicated a lag phase was quite short and difficult to predict, followed by an exponential phase of growth lasting approximately 12 days, ending up in the death phase. The whole growth curve took approximately 18 days to be completed and presented more three folds fresh cell biomass accumulation [8] reported that 0.5 g Zedoary cell culture in the presence of 3% sucrose, 13.4 µM NAA and 2.2 µM BAP showed a typical growth curve with a maximum fresh weight (approximately 6 g) after about 35 days of culture in 10 ml medium at speed of 60 cycles/minute. [12] also showed that a typical growth curve of 1 g Zedoary cell culture on the medium supplemented with 1.0 mg/l NAA. The fresh biomass reached a highest value (approximately 8 g) after more 20 days of culture in 75 ml medium at speed of 100 rpm.

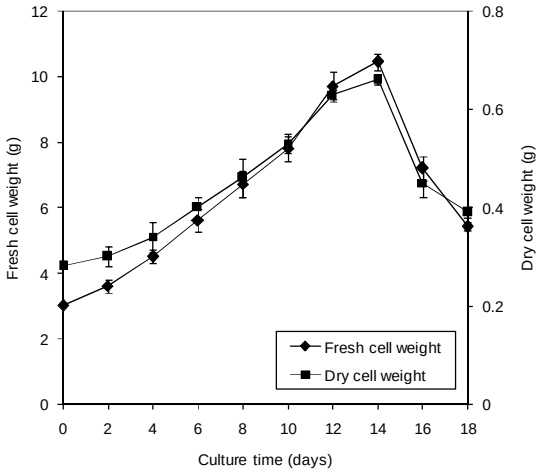

Fig 4. Biomass production of Zedoary cells on the medium with 3% sucrose, 1.5 mg/l 2,4-D and 0.5 mg/l BA ($p<0.05$).

4. Conclusion

The suspension culture offers many advantages to scale-up production of secondary metabolites in plant cells of interest. In this study, we established an efficient cell suspension culture protocol for Zedoary plant. Their suspension cells derived from secondary calli proliferated on the MS medium supplemented with 3% sucrose, 1.5 mg/l 2,4-D and 0.5 mg/l BA had a homogenous feature from a morphological viewpoint. The most important observation of this study is that suspension cells of Zedoary had a high proliferation potential and the finding will provide some basic information for the production of bioactive compounds from Zedoary cell culture in further.

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References

[1] L.D. Moi, T.M. Hoi, D.D. Huyen, T.H. Thai, N.K. Ban, *Plant resources of Vietnam-Bioactive plants*, Vol 1. Agriculture Publishing House, Ha Noi, Vietnam, 2005.

[2] W.Jr. Syu, C.C. Shen, M.J. Don, J.C. Ou , G.H. Lee, C.M. Sun, Cytotoxicity of curcuminoids and some novel compounds from *Curcuma zedoaria*, *J. Nat. Prod.* 61(1998) 1531.

[3] T. Yoshioka, E. Fuji, M. Endo, Anti-inflammatory potency of dehydrocurdione, a Zedoary-derived sesquiterpene, *Inflamm. Res.* 47 (1998) 476.

[4] H. Matsuda, T. Ninomiya, M. Yoshikawa, Inhibitory effect and action mechanism of sesquiterpenes from Zedoariae rhizoma on D-galactosamine/lipopolysaccharide-induced liver injury, *Bioorg. Med. Chem. Lett.* 8 (1998) 339.

[5] F. Bourgaud, A. Gravot, S. Milesi, E. Gontier, Production of plant secondary metabolites: a historical perspective, *Plant Sci.* 161 (2001) 839.

[6] S. Ramachandra Rao, G.A. Ravishankar, Plant cell cultures: chemical factories of secondary metabolites, *Biotechno.l Advances* 20 (2002) 101.

[7] H.S. Taha, M.K. El-Bahr, M.M. Seif-El-Nasr, *In vitro* studies on Egyptian *Catharanthus roseus* (L.) G. Don.: 1-callus production, direct shootlets regeneration and alkaloids determination, *J. Appl. Sci. Res.* 4 (2008) 1017.

[8] M.O. Mello, C.T.S. Dias, A.F. Amarai, Growth of *Bauhinia forficata*, *Curcuma zedoaria*, and *Phaseolus vulgaris* cell suspension cultures with carbon sources, *Sci. Agric.* 58 (2001a) 481.

[9] T. Murashige , F. Skoog, A revised medium for rapid growth and bioassays with tobacco tissue culture, *Physiol. Plant* 15 (1962) 473.

[10] N.H. Loc, D.T. Duc, T.H. Kwon, M.S. Yang, Micropropagation of Zedoary (*Curcuma zedoaria* Roscoe)-a valuable medicinal plant, *Plant Cell Tiss. Organ Cult.* 81 (2005) 119.

[11] M.O. Mello, M. Melo, B. Appezzato-da-Glória, Histological analysis of the callogenesis and organogenesis from root segments of *Curcuma zedoaria* Roscoe, *Braz. Arch. Biol. Technol.* 44 (2001b) 197.

[12] J.I. Miachir, V.L.M. Romani, A.F. de Campos Amaral, M.O. Mello, O.J. Crocomo, M. Melo, Micropropagation and callogenesis of *Curcuma zedoaria* Roscoe, *Sci. Agric. (Piracicaba, Braz)* 61 (2004) 427.

[13] C.B. Do, F. Cormier, Effects of low nitrate and high sugar concentrations on anthocyanin content and composition of grape (*Vitis vinifera* L) cell suspension, *Plant Cell Rep.* 9 (1991) 500.

[14] A.R. Gould, N.P. Everett, T.L. Wang, H.E. Street, Studies on the control of cell cycle in cultured plant cells: Effect of nutrient limitation and nutrient starvation, *Protoplasma* 106 (1981) 1.

[15] C.H. Wu, Y.H. Dewir, E.J. Hahn, K.Y. Paek, Optimization of culturing conditions for the production of biomass and phenolics from adventitious roots of Echinacea angustifofia, *J. Plant Biol.* 49 (2006) 193.

Nuôi cấy tế bào huyền phù của cây nghệ đen (*Curcuma zedoaria* Roscoe)

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Nghiên cứu này trình bày phương thức nuôi cấy callus và tế bào huyền phù của cây nghệ đen (*Curcuma zedoaria* Roscoe). Môi trường MS bổ sung 2% sucrose; 1,0 mg/l 2,4-D và 1,0 mg/l BA thích hợp cho nuôi cấy callus từ bẹ lá của cây nghệ đen *in vitro*. Trong quá trình nuôi cấy, các callus thứ cấp được tạo thành từ callus sơ cấp trên môi trường MS có bổ sung 0,5 mg/l 2.4-D và 0,5 mg/l BA. Các callus này có màu vàng, rắn và rời rạc. Nuôi cấy tế bào huyền phù được thiết lập với 3 g sinh khối callus tươi nuôi trong bình tam giác thể tích 250 ml, chứa 50 ml môi trường MS có bổ sung 3% sucrose; 1,5 mg/l 2,4-D và 0,5 mg/l BA với tốc độ lắc 120 vòng/phút. Sinh khối cao nhất đạt 10,44 g trọng lượng tươi (0,66 g trọng lượng khô) sau 14 ngày nuôi cấy. Các kết quả này là cơ sở cho những nghiên cứu sâu hơn về cây nghệ đen nhằm cung cấp nguồn nguyên liệu tế bào để sản xuất các hợp chất thứ cấp ở qui mô lớn.

Từ khoá: Callus, sinh khối tế bào, tế bào huyền phù, cây nghệ đen, cây thuốc.