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Saponin accumulation in cell suspension culture of *Ehretia asperula* Zollinger et Moritzi

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Abstract— **This study was intended to investigate the saponin accumulation in the cell suspension culture of Ehretia asperula Zolliger et Moritzi. Soft, friable callus was initiated and grew well in MS medium supplemented with coconut water 10%, sucrose 30g/L, BA (6-benzylaminopurine) 0.1mg/L and 2,4-D (2,4-Dichlorophenoxyacetic acid) 2-2.5mg/L, with callus induction rate 93.52% and 92.59%; callus diameter 18.33 and 17.00mm; and growth index 3.44 and 3.38, respectively, after 24 days of culture. The callus cultured in MS (Murashige & Skoog) medium added with BA 0.1mg/L and 2,4D 2.0mg/L were chosen to initiate the cell culture. The cell culturing experiments studied the effect of the agitating speed, the initial cell to medium ratio, and the addition of elicitor (methyl jasmonate)/precursor (phenylalanine) on the cell proliferation and the saponin accumulation. The cell suspension proliferated best at 140 rpm of agitation (growth index 7.81) and 40% of the initial cell to medium ratio (growth index 7.96). The highest saponin content (71.1µg/g) was detected with the use of methyl jasmonate (10mg/L) in the culture medium.**

***Keywords*— *Ehretia asperula* Zolliger et Moritzi, callus, cell suspension, saponin, 2,4-D**

I. Introduction

Cancer is the second leading cause of death worldwide. In fact, the number of global death cases from cancer is estimated to reach 9.6 million in 2018 (Bray et al., 2018). One of the most practical approaches to control this disease is cancer chemoprevention by phytochemicals. Since saponins can cause apoptosis in tumor cells, they have become valuable drugs for cancer treatment, even in clinical practice (Man et al., 2010).

Despite the rising demand for therapeutic molecules, the striking reduction of biodiversity, and the slow growth rate of plant species lead to the requirement of an alternative practice for the production of high-value plant-derived metabolites (Georgiev et al., 2009), replacing the traditional method of direct extraction from the plant. The plant cell culture has been an alternative approach with shorter culturing period and more stable biochemical content.

Ehretia asperula Zoll. et Mor., locally called –xa den|, which belongs to Boraginaceae family (Sung et al., 2009), has been used in traditional medicine in Vietnam in assisting the treatment of ulcers, tumors, and inflammation (Ly et al., 2006). This species in distributed in Vietnam, Myanmar, and Thailand (Thuy et al., 2006) and is valuable for its content of some phytochemicals, especially terpenoids with confirmed bioactivities in vitro (Kuo, 1997).

In this study, the optimum cell suspension culture condition of *Ehretia asperula* Zoll. et Mor. was investigated in order to obtain a high content of accumulated saponin.

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II. Materials and methods

A. Materials

Plant materials: In vitro explants cultured from 30cm plants of *Ehretia asperula* Zolliger et Moritzi originated from Long Bien – Hanoi – Vietnam.

Culture medium: MS (Murashige & Skoog, 1962), 1/2MS (half-strength MS), WPM (Woody Plant Medium), B5 (Gamborg B5) media. The culture media was supplemented with phytohormones including BA (6-benzylaminopurine), NAA (α-naphthaleneacetic acid), 2,4D (2,4-Dichlorophenoxyacetic acid), methyl jasmonate, phenylalanine; coconut water (10%), sucrose (30g/L), agar (0.8%) and autoclaved at 121^0C and 1 atm condition.

Culture condition: room temperature 24 ± 2^0C, relative humidity 65±2%, radiation intensity 22 µmol/m^2/s.

B. Methods

1) Callus culture

The calli were induced from in-vitro leaf pieces (1.0x1.0cm) cultured in in photoperiod 24hrs of darkness and were subcultured every three weeks. The medium was adjusted to pH 5.8 (for callus induction) or 5.6 (for callus subculture) before being autoclaved. Ascorbic acid (50mg/L) and citric acid (25mg/L) were added to the subculture media to reduce callus browning (Martin, 2006).

2) Cell suspension culture

The callus with the optimum culture medium examined from previous experiments was used to initiate the cell suspension. 3g of callus was transferred to the liquid medium (pH 5.6), and maintained at agitation (120rpm). The cell suspension with cell density of $6x10^4$ cells/ml was used in experiments 5, 6 and 7. (Cragg & Newman 2009 and Vargas & Flota, 2006)

3) Callus/Cell evaluation

The responses of callus in various media were expressed as the callus induction rate (Huang, 2015), the callus diameter, the morphology characteristics, the final fresh weight of callus after 24 days of culture, and the growth index (Sahrarro et al., 2014). The cell suspension was evaluated in terms of the cell density (10^4 cells/ml), the growth index and the saponin content (µg/g).

4) Experimental design

All experiments were completely randomize designed with three replications, and three culture vessels (4 explants/vessel) for each replication. Data were collected 24 days after being cultured and analyzed by ANOVA using SPSS 2.0 software with LSD test at $p \leq 0.05$.

Experiment 1. The effect of culture media on callus initiation: The leaf explants (1.0x1.0cm) were cultured in four different media, including MS, ½ MS, WPM and B5. All media were supplemented with BA (0.1 mg/L) and 2,4-D (2.0mg/L).

Experiment 2. The effect of coconut water on callus proliferation: The callus separated from the leaf explants cultured in the corresponding media were subcultured into MS media added with BA (0.1mg/L), 2,4-D (2.0mg/L) and coconut water (0; 5; 10; 15 and 20%), with an initial fresh weight of 0.5g/vessel.

Experiment 3. The effect of sucrose on callus proliferation: The callus separated from the leaf explants cultured in the corresponding media were subcultured into MS media added with BA (0.1mg/L), 2,4-D (2.0mg/L) and sucrose (0; 20; 30; and 40g/L), with an initial fresh weight of 0.5g/vessel.

Experiment 4. The effect of phytohormones on callus initiation and proliferation: The leaf explants (1.0x1.0cm) were cultured on MS medium supplemented with BA (0.1mg/L) and 2,4-D or NAA at different concentrations (0; 1.0; 1.5; 2.0; 2.5; and 3mg/L) to induce callus formation. In the first subculture, 0.5g callus was transferred to the corresponding subcultured media.

Experiment 5. The effect of agitating speed on the formation of cell suspension: The suspension with a cell density of $6x10^4$ cells/ml was transferred to fresh medium with an initial cell to medium ratio of 30% (v/v). The cell cultures were continuously agitated at different speeds (100; 120 and 140rpm) on a rotary shaker.

Experiment 6. The effect of the initial cell to medium ratio on cell suspension proliferation: The suspension with a cell density of $6x10^4$ cells/ml was transferred to fresh medium with different initial cell to medium ratios (10; 20; 30; 40 and 50%) and maintained in agitation at 120rpm.

Experiment 7. The effect of methyl jasmonate and/or phenylalanine on saponin accumulation: The optimal culture condition from the two previous experiments were applied in this investigation. The culture media were supplemented with methyl jasmonate (0; 5; 10; 20; and 30mg/L) and/or phenylalanine at 16.5mg/L (or 100µM).

III. Results and discussion

A. The effect of culture media on callus initiation

As can be seen from Table 1, MS medium induced the largest callus (18.00mm). Thus, the optimal macro-elements for inducing callus was MS medium. Similarly, Sharma et al. (2017) reported that the optimal medium for culturing calli from *Crataeva tapia L.* was MS medium.

B. The effect of coconut water on callus proliferation

The addition of coconut water enhanced the callus proliferation. The control sample only produced 1.62g fresh weight, with growth index 2.24, whereas coconut water at 10 and 15% resulted in 2.24 and 2.18g of fresh weight, with the growth index 3.48 and 3.36, respectively (Table 2), which were obviously higher than those of other treatments. This outcome was similar to that of Al-Khayri (2010), which cultured callus from *Pheonix dactylifera* L. species.

C. The effect of sucrose on callus proliferation

The sucrose supplementation at 30g/L resulted in a significant increase of the fresh weight, reaching 2.20g, which was 3.40 times higher than the initial (Table 3). Hence, the sucrose concentration appropriate for callus growth was 30g/L, which was similar to the studies of Frisch & Camper (1987) and Wang et al. (2012), in which, the calli from *Camellia sinensis* Wang et al. (2012) on *Panax ginseng*.

D. The effect of phytohormones on callus initiation and proliferation

The addition of BA (0.1mg/L) and 2,4-D or NAA stimulated the formation of callus (Table 4). With the addition of BA (0.1mg/L) and NAA, the callus induction rate was 100%. For 2,4-D, the concentration of 2.0 and 2.5 mg/L resulted in no statistically significant differences, with callus induction rate over 90% and callus diameter 17.00-18.33mm. After 24 days, the callus browning began.

Figure 1. Calli from leaf explants of *Ehretia asperula* Zolliger et Moritzi cultured in a) MS, b) 1/2MS, c) WPM and d) B5 media

TABLE 1. THE EFFECT OF CULTURE MEDIA ON CALLUS INITIATION

Treatments	Culture media	Callus induction rate (%)	Callus diameter (mm)	Callus formation	Morphology characteristics
1	MS	91.67 ± 2.78^a	18.00 ± 1.00^a	+++	greenish, friable, soft
2	½ MS	87.03 ± 3.21^a	14.33 ± 0.58^b	++	greenish, friable, soft
3	WPM	75.00 ± 5.56^b	15.33 ± 0.58^b	+	greenish, a little soft
4	B5	85.18 ± 1.61^a	15.33 ± 1.53^b	+	greenish, friable, a little harder

Note: The callus formation was indicated by + (present of callus), ++ (fair), and +++ (very good). Mean value in the same column with similar letter(s) are not significantly different at $p \leq 0.05$ using LSD test.

TABLE 2. THE EFFECT OF COCONUT WATER ON CALLUS PROLIFERATION AFTER 24 DAYS OF CULTURE

Treatments	Coconut water (%)	Callus fresh weight (g)	Growth index	Callus formation
Control	0	1.62 ± 0.12[a]	2.24	++
1	5	1.96 ± 0.62[b]	2.92	++
2	**10**	**2.24 ± 0.22[c]**	**3.48**	**+++**
3	**15**	**2.18 ± 0.08[c]**	**3.36**	**+++**
4	20	1.82 ± 0.06[b]	2.64	++

Note: The callus formation was indicated by + (present of callus), ++ (fair), and +++ (very good). .

TABLE 3. THE EFFECT OF SUCROSE CONCENTRATIONS ON CALLUS PROLIFERATION AFTER 24 DAYS OF CULTURE

Treatments	Sucrose (g/L)	Callus fresh weight (g)	Growth index	Callus formation
Control	Đ/C	0.62 ± 0,34[a]	0.24	-
1	20	1.84 ± 0,16[c]	2.68	++
2	**30**	**2.20 ± 0,24[d]**	**3.40**	**+++**
3	40	1.62 ± 0,09[b]	2.24	++

Note: The callus for nation was indicated by + (present of callus), ++ (fair), and ++ + (very good). .

The highest fresh weight and growth index were obtained by adding 2,4-D 2.0 and 2.5mg/L to the media, with 2.22 and 2.19g fresh weight respectively, rising by 3.44 and 3.38 times in comparing with the initial weight (Table 5). Thus, the optimal medium for callus growth was the MS medium supplemented with BA (0.1mg/) and 2,4-D (2.0 or 2.5mg/L). The results were correlative with the researches of Sahoo et al. (1997) on *Morus india* L. and those of Wang (2012) on *Panax ginseng.*

E. The effect of agitating speed on the formation of cell suspension

The rotational speed obviously affected cell proliferation. The cell culture at 140rpm resulted in highest cell density (16.33 x 10^4 cell/ml) and growth index (7.81) (Table 6). Thus, the speed of 140 rpm is suitable for culturing cell suspension of *Ehretia asperula* Zolliger et Moritzi. This outcome was quite different from that of Singh & Chaturvedi (2012) on *Spilanthes acmella* Murr. However, the agitating speed of 140 rpm used to be applied successfully in culturing cell suspension of *Catharanthus roseus* (Tanaka et al., 1988).

F. The effect of the initial cell to medium ratio on cell suspension proliferation

After the time of culture, the cell density was the highest (21.89x10^4 cells/ml), increased by 7.96 times with 40% of initial rate (Table 7). Hence, the appropriate cell to medium ratio for cell culture of *Ehretia asperula* Zolliger et Moritzi was 40%, which was applied in the further experiment.

TABLE 4. THE EFFECT OF PHYTOHORMONES ON CALLUS INITIATION

Treatments	Phytohormones (mg/L)		Callus induction rate (%)	Callus diameter (mm)	Callus formation	Morphology characteristics
Control	0.0		-	-	-	No callus formation
	2,4D	BA				
1	1.0	0.1	89.82 ± 1.61[b]	16.33 ± 1.53[ab]	++	Greenish, soft, friable
2	1.5	0.1	77.78 ± 2.78[a]	14.33 ± 1.15[a]	++	Greenish, soft, friable
3	2.0	0.1	93.52 ± 4.24[b]	18.33 ± 0.58[b]	+++	Greenish, soft, friable
4	2.5	0.1	92.59 ± 3.20[b]	17.00 ± 1.00[b]	+++	Greenish, soft, friable
5	3.0	0.1	-	-	-	No callus formation
	NAA					
6	1.0	0.1	100.00 ± 0.00[c]	16.33 ± 1.53[ab]	+++	Yellow, hard, compact
7	1.5	0.1	100.00 ± 0.00[c]	18.00 ± 1.73[b]	+++	Yellowish, hard, compact
8	2.0	0.1	100.00 ± 0.00[c]	18.33 ± 1.53[b]	+++	Yellowish, hard, compact
9	2.5	0.1	100.00 ± 0.00[c]	17.33 ± 1.00[b]	+++	Yellowish, hard, compact
10	3.0	0.1	100.00 ± 0.00[c]	18.33 ± 1.53[b]	+++	Yellowish, hard, compact

Note: The callus formation was indicated by + (present of callus), ++ (fair), and +++ (very good).

TABLE 6. THE EFFECT OF AGITATING SPEED ON THE FORMATION OF CELL SUSPENSION AFTER 24 DAYS OF CULTURE

Treat-ments	Agitating speed (rpm)	Cell density (x10^4 cells/ml)	Growth index	Cell suspension formation
1	100	7.67 ± 0.34[a]	3.14	++
2	120	11.89 ± 0.19[b]	5.42	+++
3	140	16.33 ± 0.67[c]	7.81	+++

Note: The cell suspension formation was indicated by + (present), ++ (fair), and +++ (very good).

TABLE 8. THE EFFECT OF THE INITIAL CELL TO MEDIUM RATIO ON THE CELL SUSPENSION PROLIFERATION AFTER 24 DAYS OF CULTURE

Treat-ments	Initial Cell to medium ratio (%)	Cell density (x10^4 cells/ml)	Growth index	Cell suspension formation
1	10	1.00 ± 0.11[a]	0.35	+
2	20	3.89 ± 0.19[b]	2.29	+
3	30	12.00 ± 0.67[c]	5.88	+++
4	40	21.89 ± 0.84[d]	7.96	+++
5	50	7.89 ± 0.70[e]	1.59	+

Note: The cell suspension formation was indicated by + (present), ++ (fair), and +++ (very good).

TABLE 5. THE EFFECT OF PHYTOHORMONES ON CALLUS PROLIFERATION AFTER 24 DAYS OF CULTURE

Treat-ments	Phytohormones (mg/L)		Callus fresh weight (g)	Growth index
Control	0.0		-	-
	2,4D	BA		
1	1.0	0.1	1.10 ± 0.09^{a}	1.20
2	1.5	0.1	2.00 ± 0.04^{c}	3.01
3	2.0	0.1	2.22 ± 0.14^{d}	3.44
4	2.5	0.1	2.19 ± 0.02^{d}	3.38
5	3.0	0.1	-	-
	NAA			
6	1.0	0.1	1.36 ± 0.06^{b}	1.71
7	1.5	0.1	1.32 ± 0.01^{b}	1.64
8	2.0	0.1	1.25 ± 0.05^{b}	1.50
9	2.5	0.1	1.27 ± 0.06^{b}	1.55
10	3.0	0.1	1.26 ± 0.03^{b}	1.53

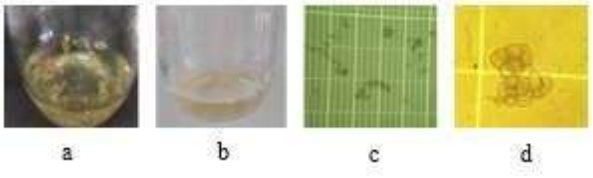

Figure 5. a) 3g of callus was transferred to liquid media to initiate cell suspension, b) Cell suspension cultured at 140 rpm with the initial cell to medium ratio of 40% after 24 days, c) Cells observed under the microscope, d) Cell clumps

G. The effect of methyl jasmonate and/or phenylalanine on saponin accumulation

Saponin was not detected in the in-vitro explants and callus of *Ehretia asperula* Zolliger et Moritzi cultured on MS media supplemented with BA (0.1mg/L) and 2,4-D (2.0mg/L) but was present in the cell suspension (41.1 µg/g). The control sample showed the highest cell density (16.44×10^4 cells/ml) and growth index (5.73) (Table 8). The addition of methyl jasmonate and phenylalanine had a significant effect on cell proliferation and the total saponin accumulation of the suspension. With the supplementation of methyl jasmonate (10mg/L), although cell proliferation decreased, the total saponin content increased significantly, reaching 71.1µg/g. Furthermore, by comparing the effects of methyl jasmonate alone and in combination with phenylalanine, it can be seen that methyl jasmonate predominated phenylalanine in stimulating saponin accumulation. Correlative results could be seen in the study of Wang et al. (2012) on *Panax gingseng* and Hayashi et al. (2003) on *Glycyrrhiza glabra* cell culture.

IV. Conclusion

The optimal medium for callus and cell culture of *Ehretia asperula* Zolliger et Moritzi were MS medium supplemented with coconut water (10%), sucrose (30g/L), BA (0.1mg/L) and 2,4-D (2.0mg/L). The appropriate rotational speed for cell proliferation was 140 rpm. The highest growth index was obtained with the initial cell to medium ratio of 40%. Methyl jasmonate predominated phenylalanine in enhancing saponin accumulation in the cell suspension. The total saponin content increased significantly when methyl jasmonate 10mg/L was added to the culture medium.

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TABLE 7. THE EFFECT OF METHYL JASMONATE AND/OR PHENYLALANINE ON SAPONIN ACCUMULATION IN 24 DAYS OF CULTURE

Treatments	Methyl jasmonate (mg/L)	Phenylalanine (mg/L)	Cell density ($x10^4$ cells/ml)	Growth index	Total saponin content (µg/g)
Control	0	0	16.44 ± 1.26^{e}	5.73	41.1
1	5	0	12.11 ± 0.70^{d}	3.96	58.2
2	10	0	8.55 ± 0.39^{c}	2.50	71.1
3	20	0	4.33 ± 0.34^{a}	0.77	26.1
4	30	0	5.78 ± 0.51^{b}	1.36	36.4
5	10	16.5	11.56 ± 0.5^{d}	3.73	55.6
6	0	16.5	5.56 ± 0.51^{b}	1.27	44.5

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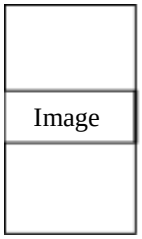

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