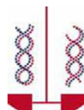

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Regulation and improvement of triterpene formation in plant cultured cells of *Eriobotrya japonica* Lindl

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Loquat (*Eriobotrya japonica* Lindl) is a traditional Chinese medicinal plant that contains triterpenes, which have been shown to exhibit pharmaceutical activities. In this study, we investigated various different culture conditions for cultured cells of loquat to produce triterpenes, including illumination, carbon source, nutrient composition and culture system. When cultured on 2.5 mg/l of 6-benzyladenine, 1 mg/l of naphthalene acetic acid and 30 g/l of sucrose at 25 ± 2 °C in the dark for 30 days, the nutrient composition significantly regulated the cell growth and triterpene production. Supplied with the Murashige and Skoog medium reached higher level of dry weight (1.27 ± 0.09 g per flask) and total triterpene production (151.54 ± 12.58 mg/g of cultured cells), and the N6 medium produced tormentic acid but inhibited other triterpene products, while the B5 medium produced relatively high corosolic acid. Also found, suspension cultures of loquat cell could achieve high productivity as callus culture.

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[**Key words:** Loquat; Triterpenes; Tissue culture; Secondary metabolite; Suspension culture]

The loquat (*Eriobotrya japonica* Lindl), which belongs to the Rosaceae family, is a common fruit tree that is widely distributed in Taiwan and China. Loquat leaves are used in traditional Chinese medicine to treat cough and chronic bronchitis. The leaf contains a variety of triterpenes, including tormentic acid (TA), corosolic acid (CA), ursolic acid (UA), maslinic acid (MA), and oleanolic acid (OA) (Fig. 1) (1). Several studies have demonstrated that these triterpenes possess many pharmaceutical effects such as anti-oxidation (2), anti-inflammation (3), anti-tumor (4), hepatoprotection (5), anti-hypertension (6), anti-diabetes (7,8) obesity (9) and reduced vascular smooth muscle (10). Since triterpenes offer a wide variety of pharmaceutical effects, the importance of these bioactive compounds has in recent years resulted in great interests.

Plant cell cultures have been successfully applied to produce large quantities of secondary metabolites from many plants. It has been reported that callus tissue culture of *E. japonica* was able to produce large amounts of triterpenes (11). However, the optimum culture conditions and the approach for constantly producing large quantities of triterpenes in cell culture still remain unknown. In plant cell culture, various approaches such as illumination and changing nutrient supply can improve the yield of the cell biomass and regulate the secondary metabolites. Recent developments showed that manipulation of the culture environment is effective in increasing the product accumulation. Moreover, the nutrient level is an easy approach to influence the expression of many secondary metabolite pathways (12).

In plant cell culture, cells are usually grown with simple sugars as carbon source in the medium for the energy resource. The fact is that the level of sucrose has been shown to affect the productivity of secondary metabolites in many plants (13,14). Furthermore, the inorganic elements in medium are as important as carbon source. These elements influence the cell growth and division by regulating the protein, gene expression and other pathways (15). The levels of elements can affect the cell growth and secondary metabolite production individually, or interact in a ratio such as ammonium/nitrate (12) and magnesium/calcium (16). The many kinds of media which were developed for various cultures are considerably different at regulating the cell growth and secondary metabolites product.

Recently, suspension culture was considered that a key progress to achieve commercial scale (17). The liquid medium allows the close contact with the tissue which stimulates and facilitates the uptake of nutrients and hormones (18), leading to better cell growth. Researchers developed many models to achieve large scale and high productivity for commerciality such as shikonin in *Lithospermum erythrorhizon* cell line (19) and paclitaxel in *Taxus brevifolia* (20).

In this study, we evaluated the cell growth and triterpene content of calli from different tissues of *E. japonica* Lindl. We studied the

Abbreviations: BA, 6-Benzyladenine; NAA, Naphthalene acetic acid; HPLC, High performance liquid chromatography; TA, Tormentic acid; MA, Maslinic acid; CA, Corosolic acid; OA, Oleanolic acid; UA, Ursolic acid.

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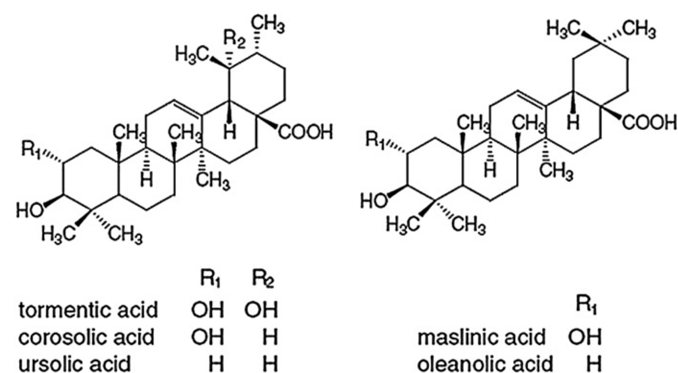

FIG. 1. Structure of the triterpenes in *E. japonica.*

effects on triterpene production from harvest time, light, medium formulation and sucrose concentrations on triterpene production (TA, MA, CA, OA, and UA). Additionally we also investigated the potential of the suspension culture in triterpene production.

MATERIALS AND METHODS

Callus culture establishment Seeds of *E. japonica* Lindl were provided by Mr. Chen-I Chen, Department of Bio-industry and Agribusiness Administration in Taiwan. The seed surfaces were sterilized in 70% (v/v) ethanol for 1 min, followed by 20 min in 180 ml of 1% (w/v) sodium hypochlorite supplemented with two drops of Tween 20, and rinsed three times with sterile distilled water. The seeds were then placed on the Murashige and Skoog (MS) basal medium (21) containing 3% (w/v) sucrose and 0.3% (w/v) gelrite. One month later, the leaves, stems and roots were harvested and weighed separately from the culture medium. Each tissue was then sliced into 2- to 3-mm slices and transplanted into MS medium supplemented with 2.5 mg/l 6-benzyladenine (BA) and 1 mg/l 1-naphthalenacetic acid (NAA) for callus induction. The pH of the medium was adjusted to 5.8 and autoclaved at 121 °C for 20 min. The calli were all grown at 25 ± 2 °C in the dark and subcultured every 20 days.

Callus induction from different tissues of *E. japonica* Lindl The 20-day-old callus (about 1.5 g) induction from leaves, stems and roots of *E. japonica* Lindl was transferred to a 400 ml Erlenmeyer flask (with flask bottom diameter 10 cm) containing 100 ml MS medium supplemented with 2.5 mg/l BA, 1 mg/l NAA and 3% (w/v) sucrose. The calli were then grown at 25 ± 2 °C in the dark for 30 days. All fresh calli were collected and then dried at 60 °C for 48 h to determine triterpene content. A time course experiment was also performed; the calli were harvested every 3 days for 40 days to measure the weight and triterpene content. All experiments were repeated 10 times.

Maximization of triterpene production in leaf calli To study the effect of light, the leaf callus culture was also incubated under a light intensity of 1000 lx (cool white fluorescent light, Philips, Holland), with 16/8 photoperiod condition in MS medium supplemented with 2.5 mg/l BA, 1 mg/l NAA and 3% (w/v) sucrose for 30 days. To study the effect of the medium, leaf calli were grown in MS, LS (22), B5 (23), W (24), N6 (25) or SH (26) supplemented with 2.5 mg/l BA, 1 mg/l NAA and 3% (w/v) sucrose at 25 ± 2 °C in the dark for 30 days. To study the effects of sucrose concentrations, the leaf calli were grown in MS medium supplemented with different concentrations (10, 20, 30, 40 or 50 g/l) of sucrose and containing 2.5 mg/l BA and 1 mg/l NAA at 25 ± 2 °C in the dark for 30 days. All fresh calli were collected and dried at 60 °C for 48 h to determine triterpene content.

Initiation of suspension cultures Callus cultures were maintained by subculturing every 20 days and used for initiating cell suspension cultures. Suspension cultures were established by inoculating the 3 g of 20-day-old callus masses in 1-L Erlenmeyer flasks containing 400 ml liquid MS medium. These cultures were incubated on a rotary shaker at 120 rpm. The temperature was maintained at 25 ± 2 °C in dark. Suspension cultures were sampled 10 ml at an interval of 2 days.

Determination of triterpene content Triterpenes were extracted from 0.3 g of dried cell with 20 ml of 95% ethanol at 70 °C for 8 h, three times. The combined ethanolic extract was filtered through filter papers (Qualitative filter paper No.1, Advantec, Japan) and the filtrate was concentrated under reduced pressure (centrifugal evaporator CVE 3100, Eyela, Japan). About 50 mg of the condensed extract was redissolved in 10 ml of methanol/water (85:15) under 20-min sonication to ensure the complete extraction of triterpenes, and filtered through a 0.45-μm membrane (Millex-HV 0.45 μm, PVDF, 13 mm, Millipore, USA). Then, 20 μl of the filtered extract was subjected to HPLC in triplicate. The triterpene content was determined by HPLC using the conditions reported by Ho et al. (1). In brief, HPLC was performed on a Shimadzu 10A system equipped with one pump (LC-10AT Shimadzu, Japan) and an RI spectrophotometric detector (RID-10A, Shimadzu, Japan). The mobile phase (methanol: 0.15% aqueous acetic acid = 85:15) was pumped at a flow rate of 0.5 ml min^{-1} with a HyPURITY C-18, <phi>4.6 × 250 mm HPLC column. An oven (CTO-10AS, Shimadzu, Japan) was used in this experiment to keep the column at 35 °C. The cycle time of analysis was 40 min (Supplementary Fig. S1).

Statistical analysis All experiments were repeated 10 times and the values are presented as means ± standard deviation (SD). The data for different tissues, media and sucrose concentrations were statistically analyzed using one-way analysis of variance and the means were compared by Duncan's multiple range tests at a 5% probability level.

RESULTS AND DISCUSSION

Triterpene content in different tissues of *E. japonica* Lindl The accumulation of triterpenes in different tissues was investigated, as summarized in Table 1. After 30 days of cultivation, the dry weight of the callus induced from leaves reached 1.27 ± 0.09 g/l, which was the heaviest among the tissues examined. Furthermore, the total triterpene content in the leaf callus was the highest (151.54 ± 12.58 mg/g) (Supplementary Fig. S2). Of the five triterpenes evaluated, TA (63.80 ± 13.7 mg/g) was the main component, followed by CA (47.34 ± 4.93 mg/g) and MA (21.91 ± 1.33 mg/g). Based on Ho's study (1), the highest total triterpene content of *E. japonica* Lindl plants in Taiwan was 15.7 ± 0.3 mg/g (Supplementary Figs. S3 and S4). Thus, the cultured leaf calluses produced significantly greater quantities of triterpenes compared with plant materials.

Triterpene content in leaf calli during growth The growth curve and triterpene production of leaf calli were recorded by dry weight of cells and the amount of five different triterpenes, as shown in Fig. 2. The growth rate of leaf callus started to rise on the 12th day of culture and reached a maximum at 1.35 ± 0.15 g per flask after 35 days of culture. The dry weight of the callus increased by 12.6-fold compared with the initial value. All five triterpenes showed a similar production curve and were positive correlated with callus growth. The total production of triterpenes started to rise on the 24th day of culture and increased rapidly until day 30 reaching the maximal amount of 151.54 ± 12.58 mg/g of the cultured cells. The production of each triterpenes increased by 2.2- to 3.3-fold compared with their initial values. The TA content was the greatest of the five triterpenes but the increase in TA production was the lowest (2.2-fold). After 30 days of culture, the total triterpene content dropped gradually. These data indicate that triterpenes accumulate rapidly from day 24 to day 30 when the leaf callus culture entered the stationary phase.

The effect of light on triterpene production in leaf calli It has been shown that calli grown in the dark produce less chlorophyll, which would be favorable for triterpene production (11). It has also been reported that culturing *Calendula officinalis* L. with 0.1 mg/l 2,4-dichlorophenoxyacetic acid and 0.5 mg/l 6-(γ,γ-dimethylallylamino)-purine could stimulate more OA production in the dark, whereas

TABLE 1. Yield of triterpenes obtained from callus cultures induced from various tissues of *E. japonica.*

Tissue	Dry weight per flask (g) *	Triterpene content (mg/g of cultured cell) *					
		TA	MA	CA	OA	UA	Total
Leaf	1.27 ± 0.09[a]	63.80 ± 13.70[a]	21.91 ± 1.33[a]	47.34 ± 4.93[a]	2.90 ± 0.53[a]	15.56 ± 1.79[a]	151.54 ± 12.58[a]
Stem	1.20 ± 0.08[a]	43.01 ± 5.60[b]	18.24 ± 4.56[b]	38.34 ± 6.62[b]	2.78 ± 0.93[a]	12.78 ± 1.84[b]	115.18 ± 15.66[b]
Root	1.12 ± 0.12[b]	28.85 ± 4.63[c]	14.21 ± 4.95[c]	28.32 ± 4.25[c]	1.86 ± 0.34[b]	9.27 ± 0.92[c]	82.53 ± 13.08[c]

Values are means ± SD.
* Means within a column followed by the same letters are not significantly different at the 5% level by Duncan's test.

supplementing the culture medium with 0.4 mg/l 2,4-dichlorophenoxyacetic acid and 0.4 mg/l kinetin increased OA synthesis under light (27). Therefore, the hormone composition in the culture medium would influence the effect of light on the biosynthesis of OA. However, how light affects triterpene biosynthesis is still unclear. Therefore, in this study, we measured the amount of five triterpenes after exposing the calli to light and dark growth conditions (Table 2). The leaf callus culture was incubated under a light intensity of 1000 lx or in the dark for 30 days. The total triterpene content in the light condition (100.23 ± 23.15 mg/g of the cultured cells) was much less than that in the dark (151.54 ± 12.58 mg/g of the cultured cells). The biosynthesis of each triterpenes was less under light. Thus, darkness was beneficial for leaf callus to produce triterpenes and was more favorable for triterpene purification by reducing the amount of chlorophyll in the culture.

The effect of media on triterpene production in leaf calli We investigated the triterpene content of leaf calli transplanted into six different media, MS, LS, B5, W, N6 and SH (Table 3), which vary in their mineral nutrient and vitamin contents. The composition of media significantly affected both the dry weight of the calli and triterpene production. The MS and LS media contained the most suitable growth components to stimulate callus growth. The dry weights of the calli were 1.27 ± 0.09 g per flask and 1.22 ± 0.30 g per flask in the MS and LS media, respectively. These media contain greater mineral contents than the other media. These data suggest that the greater mineral concentration better stimulated callus growth. However, the LS medium did not induce triterpene synthesis (60.30 ± 6.75 mg/g of the cultured cells) as much as the MS medium (151.54 ± 12.58 mg/g of the cultured cells). Thus, the growth of calli and the total triterpene production was not closely correlated. Compared with the LS medium, the MS medium contains vitamin B3 and B6, which are necessary for the synthesis of triterpenes. This could explain why calli growing in the MS medium produced greater amounts of triterpenes.

Even though the dry weight of callus grown in the W medium was only 0.83 ± 0.11 g per flask, the total triterpene content reached 122.09 ± 18.82 mg/g of the cultured cells. Therefore, although the low macronutrient content in the W medium did not aid callus growth, the medium did stimulate triterpene synthesis. Of note, the triterpene content was markedly different between the five media. The calli grown in the B5 medium contained much greater amounts of CA (40.65 ± 12.40 mg/g of the cultured cells), whereas N6 medium produced TA (58.45 ± 12.11 mg/g of the cultured cells) majorly and the other triterpene production were inhibited. These results indicate that even though triterpenes share similar chemical structures, different growth media can change the biosynthesis ratio of the individual triterpenes.

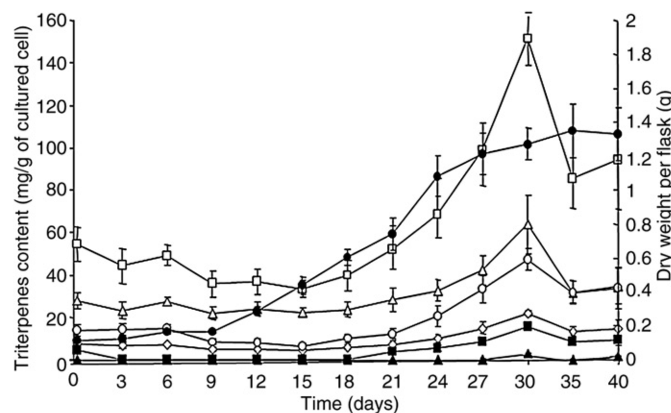

FIG. 2. Time-course of cell growth and triterpene production in leaf callus culture of *E. japonica* (open triangles, TA; open diamonds, MA; open circles, CA; closed triangles, OA; closed squares, UA; open squares, total triterpenes; closed circles, dry weight). Vertical bars indicated standard error.

Effect of the sucrose concentration on triterpene production in leaf call In suspension culture of *Uncaria tomentosa*, the optimal sucrose concentration for synthesizing UA and OA ranged from 20 to 50 g/l (28). Therefore, we investigated the effect of the sucrose concentration on growth and triterpene production of the leaf callus. As shown in Table 4, the dry weight of callus increased with increasing sucrose concentration. The dry weight of the callus reached 1.58 ± 0.19 g per flask when the sucrose concentration was 50 g/l. This indicates that sucrose is an essential nutrient for callus growth. When the calli were grown in medium with a sucrose concentration of 10 g/l, the dry eight of callus was decreased by 3.2-fold as compared with that at 30 g/l sucrose. However, the total triterpene content in the leaf callus was not positively correlated with the sucrose concentration. The maximum total triterpene content (151.54 ± 12.58 mg/g of the cultured cells) and that of the individual triterpenes was observed at the sucrose concentration of 30 g/l. There was no detectable OA in the callus culture at the sucrose concentrations of 10, 40 and 50 g/l. It is worth noting that, when the sucrose concentration in the culture medium increased from 10 to 20 g/l, unlike the dry weights of callus, the triterpene content did not increase proportionally to the sucrose concentration. Furthermore, when the sucrose concentration exceeded 30 g/l, the triterpene content in the leaf callus decreased. These results are different from those of a previous paper (28) in which the terpene content was measured. Therefore, to achieve the maximum rate of total triterpene production, 30 g/l sucrose should be added to the culture medium.

Time course of biomass growth and triterpenes in suspention culture The time course of biomass growth and secondary metabolites in cell suspension cultures of leaf callus were shown in Fig. 3. It is concluded that MS medium supplemented with 2.5 mg/l BA, 1 mg/l of NAA, and 30 g/l of sucrose at 25 ± 2 °C in the dark was efficient to achieve high production. Therefore, this result was selected to investigate the suspension culture. In this suspension culture, exponential growth of biomass was followed during 8–18 days. Maximum biomass growth in suspension culture (17 ± 2.7 g/l) and the maximum total triterpene content (149 ± 2.28 mg/g of the cultured cells) were observed at 18 and 26 days after cultivation, respectively. The efficiency of suspension system has been demonstrated previously (29). The growth of plant cells is more rapid in suspension than in callus culture and is more readily controlled because the culture medium can easily be amended or changed (30). The results showed that the biomass growth and secondary metabolite production in suspension culture was as well as the callus tissue culture. However, the suspension culture is able to make the culture period shorter.

In conclusion, this is the first report to describe the optimization of triterpene production in the cell culture of *E. japonica*. We found that the leaves were the best plant tissue to induce calli to produce triterpenes. Furthermore, light was not needed for the biosynthesis of triterpenes in the callus, which was favorable for triterpene purification by reducing the amount of chlorophyll in the culture. We also found that the composition of the culture medium plays an important role in affecting triterpene production in the callus. However, factors stimulating calli growth appear to be different from those which induce triterpene production. Minerals and sucrose stimulated growth of the leaf calli, but did not directly affect triterpene synthesis. Nevertheless, vitamins could induce both callus growth and triterpene production.

Therefore, a balance between nutrients that stimulate cell growth and those that induce triterpene production is very important for manufacturing triterpenes by calli. Though the five triterpenes from loquat have similar structure, one of the problems would be the purification of these five triterpenes. However, this study showed that, by means of nutrient composition control, it is possible to regulate and improve the triterpene production, and will be helpful

TABLE 2. Effect of light on cell growth and triterpene production in the leaf calli of *E. japonica*.

Light	Dry weight per flask (g)	Triterpene content (mg g^{-1} dry weight)					
		TA	MA	CA	OA	UA	Total
Dark	1.27 ± 0.09	63.80 ± 13.70	21.91 ± 1.33	47.34 ± 4.93	2.90 ± 0.53	15.56 ± 1.79	151.54 ± 12.58
Light	1.17 ± 0.19	49.18 ± 12.37	14.34 ± 3.46	26.60 ± 12.47	1.84 ± 2.16	8.26 ± 2.16	100.23 ± 23.15

Values are means ± SD of 10 replicate analyses.

TABLE 3. Yield of triterpenes obtained from the leaf callus culture incubated in various culture media.

Medium	Dry weight per flask (g) *	Triterpene content (mg/g of cultured cell) *					
		TA	MA	CA	OA	UA	Total
MS	1.27 ± 0.09[a]	63.80 ± 13.70[a]	21.91 ± 1.33[a]	47.34 ± 4.93 0[a]	2.90 ± 0.53[a]	15.56 ± 1.79[a]	151.54 ± 12.58[a]
LS	1.22 ± 0.30[a]	23.80 ± 7.91[c]	10.10 ± 1.18[d]	20.14 ± 4.58[c]	n.d	6.24 ± 1.26[d]	60.30 ± 6.75[d]
B5	1.01 ± 0.15[b]	21.46 ± 4.66[c]	13.25 ± 3.57[c]	40.65 ± 12.40[b]	2.53 ± 0.56[b]	9.99 ± 2.87[c]	87.89 ± 23.34[c]
W	0.83 ± 0.11[c]	48.20 ± 8.82[b]	17.02 ± 2.71[b]	44.98 ± 8.86[ab]	n.d	11.88 ± 1.25[b]	122.09 ± 18.82[b]
N6	0.84 ± 0.09[c]	58.45 ± 12.11[a]	10.68 ± 2.00[d]	17.65 ± 3.59[c]	n.d	6.66 ± 2.53[d]	93.45 ± 18.38[c]
SH	0.93 ± 0.11[bc]	27.7 ± 8.81[c]	5.31 ± 2.19[e]	7.46 ± 3.29[d]	n.d	4.33 ± 1.15[e]	44.80 ± 14.99[e]

n.d = not detected.
Values are means ± SD.
* Means within a column followed by the same letters are not significantly different at the 5% level by Duncan's test.

TABLE 4. Effect of sucrose concentration on growth and triterpene production in leaf calli of *E. japonica*.

Sucrose concentration	Dry weight per flask (g) *	Triterpene content (mg/g of cultured cell) *					
		TA	MA	CA	OA	UA	Total
10 g l^{-1}	0.40 ± 0.05[d]	53.91 ± 8.54[ab]	14.37 ± 1.88[c]	20.24 ± 3.87[c]	n.d	6.07 ± 1.48[b]	94.61 ± 14.68[bc]
20 g l^{-1}	0.89 ± 0.13[c]	45.86 ± 8.56[bc]	17.54 ± 2.57[b]	33.77 ± 7.10[b]	1.76 ± 0.39[b]	9.54 ± 1.54[b]	108.49 ± 15.6[b]
30 g l^{-1}	1.27 ± 0.09[b]	63.80 ± 13.70[a]	21.91 ± 1.33[a]	47.34 ± 4.93[a]	2.90 ± 0.53[a]	15.56 ± 1.79[a]	151.54 ± 12.58[a]
40 g l^{-1}	1.31 ± 0.18[b]	41.46 ± 10.19[c]	15.15 ± 3.64[bc]	29.71 ± 7.03[b]	n.d.	8.96 ± 1.56[b]	95.30 ± 19.87[bc]
50 g l^{-1}	1.58 ± 0.19[a]	45.73 ± 13.43[c]	12.96 ± 3.70[c]	19.45 ± 5.56[c]	n.d.	7.34 ± 1.81[b]	85.50 ± 21.31[c]

n.d = not detected.
Values are mean ± SD.
* Means within a column followed by the same letters are not significantly different at the 5% level by Duncan's test.

for further application. Moreover, tissue culture of *E. japonica* Lindl in suspension culture displayed high production.

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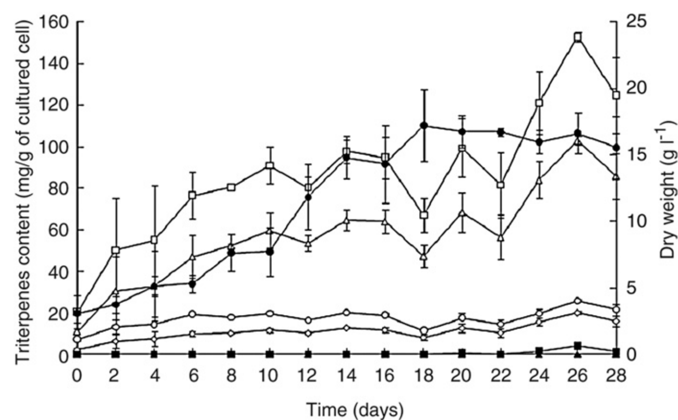

FIG. 3. Time-course of cell growth and triterpene production in suspension culture of *E. japonica* cells (open triangles, TA; open diamonds, MA; open circles, CA; closed triangles, OA; closed squares, UA; open squares, total triterpenes; closed circles, dry weight). Vertical bars indicated standard error.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jbiosc.2010.06.009.

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