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Simultaneous production of anthocyanin and triterpenoids in suspension cultures of *Perilla frutescens*

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Abstract

When cultivated in Murashige & Skoog medium supplemented with 0.2 mg l^{-1} 2,4-dichlorophenoxy acetic acid and 0.5 mg l^{-1} 6-benzyladenine, *Perilla frutescens* cells in suspension culture grew rapidly reaching about 13.6 g dry wt l^{-1} after 12 days. The cell line produced both anthocyanin 0.9 g l^{-1} and triterpenoids: 16 mg l^{-1} oleanolic acid (OA), 25 mg l^{-1} ursolic acid (UA) and 14 mg l^{-1} tormentic acid (TA). When *P. frutescens* cells of 7-day-old cultures were exposed to a yeast elicitor at 0.5–5% (v/v) for 7 days, it was found that anthocyanin content peaked at 10.2% of dry weight with yeast elicitor at 1% (v/v) whereas the maximum production of oleanolic acid and ursolic acid in cultures treated with 2% (v/v) yeast elicitor was 19 and 27 mg l^{-1}, a 46 and 24% increase over the control, respectively. This is the first report of simultaneous production of both anthocyanin and triterpenoids in a single culture system.

Keywords: Cell suspension culture; Elicitation; *Perilla frutescens*; Labiatae; Anthocyanin; Triterpenoids

1. Introduction

Perilla frutescens (L.) Britt. (Labiatae) is an edible plant frequently used as one of the most popular garnishes and food colorants in some Asian countries such as China and Japan. The leaf and (or) stem of the herb, shown to be detoxicant, anti-tussive, antibiotic and antipyretic, is being also utilised as a folk medicine for treating intestinal disorders and allergies particularly in the traditional Chinese medical practice [1]. Subsequent to our investigation of the composition and anti-fungal activity of essential oils extracted from *P. frutescens* leaves [2], we reported recently an efficient procedure for quantifying the three main bioactive triterpenes tormentic acid (TA), oleanolic acid (OA) and ursolic acid (UA) (Fig. 1) in leaves of wild *P. frutescens* [3]. Concerning the bioactivity of the three acids, TA was ascertained to be an anti-inflammatory and DNA polymerase-inhibitory phytochemical [4] while OA possesses hepatoprotective and anti-ulcer potentials, and UA exerts its anti-tumor action through enhancing the production of both nitric oxide and tumor necrosis factor-α [5].

Cell culture, emerged as a viable route for biosynthesising phytochemicals, provides a model for the production of pharmacologically important natural products such as artemisinin and paclitaxel characterised originally from plants. Although bioreactor-based systems for the scaled-up production of anthocyanin pigments as red food colouring agents using suspended cell cultures of *P. frutescens* have been described as an alternative to synthetic materials [6], little information is available up to date about the pharmacologically significant components in the cultured *P. frutescens* cells. As a continuation of our interest in the in vitro production of medically important secondary metabolites from Chinese traditional medicinal herbs [7], we tried for the simultaneous production of anthocyanin and the bioactive triterpenoids in a single culture system of *P. frutescens* with additional attention paid to the effect of yeast elicitor in the cultures.

2. Materials and methods

2.1. Plant material

Applied as an explant source was *P. frutescens* collected in June 2002 from the suburb of Nanjing, China with its voucher specimen (NJU-070801) identified by Associate Prof. L.X. Zhang deposited in the Herbarium of Nanjing University.

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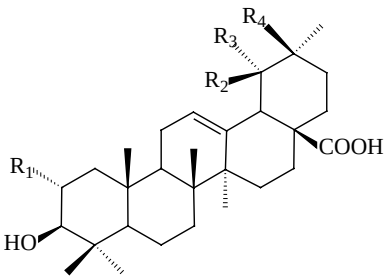

tormentic acid: R_1=R_3=OH, R_2=CH_3, R_4=H
oleanolic acid: R_1= R_2= R_3=H, R_4= CH_3
ursolic acid: R_1= R_3= R_4=H, R_2= CH_3

Fig. 1. Chemical structures of three triterpenes found in the cell cultures of *Perilla frustescens*.

2.2. Initiation and maintenance of suspension cultures

Leaves, cleaned with running water for about 30 s, were disinfected successively using detergent (1 min), 70% (v/v) ethanol (1 min) and 0.5% (v/w) sodium hypochloride (10 min) containing Tween 20 (three drops per 100 ml) followed by being rinsed twice in sterile water for 5 min. Leaf segments (ca. 5 mm long) were excised and cultured in the dark in 100 mm × 15 mm sterile petri dishes containing 25 ml of Murashige & Skoog (1962) medium (MS) supplemented with 0.5 mg l^{-1} 6-benzyladenine and 2.0 mg l^{-1} α-naphthaleneacetic acid [8]. Calli emerging on the leaf explants were excised and cultivated on the same medium at 25 °C for additional 1-month period.

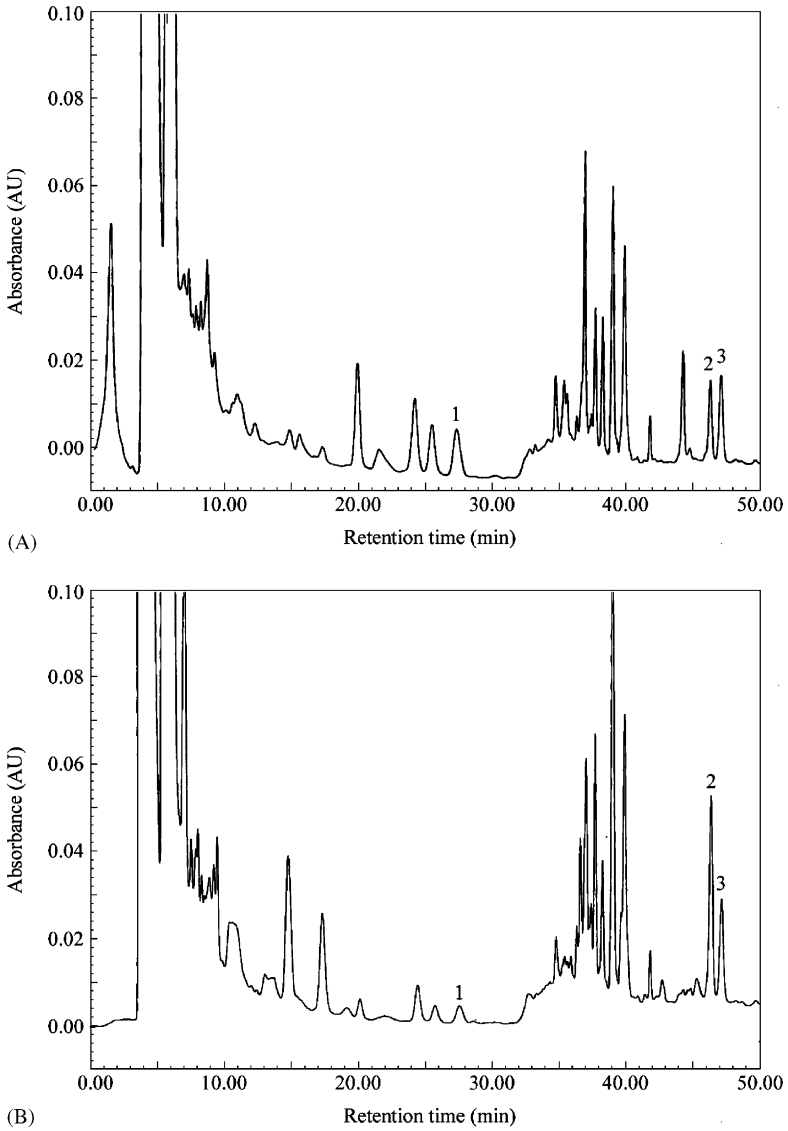

Fig. 2. Chromatogram of the methanol extracts from elicited *Perilla frustescens* cell aggregates (B) and the control (A). Peaks: 1, tormentic acid; 2, oleanolic acid and 3, ursolic acid. Seven-day-old cell cultures were treated with yeast elicitor at 1% (v/v) for 3 days. Control received the same volume of water only.

Callus tissues of the selected strain PF3 were used to initiate suspension cultures by transferring 2.5 g of friable callus into 500-ml flasks containing 100 ml of MS medium supplemented with 0.2 mg l^{-1} 2,4-dichlorophenoxy acetic acid, 0.5 mg l^{-1} 6-benzyladenine and 30 g l^{-1} sucrose. The cultures were kept on a rotary shaker at 120–130 rpm and maintained at 25 °C under 16 h light per day.

2.3. Elicitor preparation

A carbohydrate fraction isolated from the yeast extract was prepared by ethanol precipitation as described by Chen and Chen (2000) [9]. Briefly, 50 g of the yeast extract (Sigma, USA) was dissolved in 250 ml of distilled water. Ethanol was added to 80% (v/v). The precipitate was allowed to settle for 4 days at 6 °C and the supernatant was decanted and discarded. The gummy precipitate was dissolved in 250 ml of distilled water. The ethanol precipitation was repeated. The second ethanol precipitate was dissolved in 200 ml of distilled water, yielding the crude preparation that was used after being filtered through a 0.45-μm filter. The yeast elicitor preparations were added to 7-day-old cell cultures at different amounts (0–5.0 ml). For the control, 5 ml of distilled water was added to each flask.

2.4. Analyses

Biomass was quantified in terms of dry weight, and anthocyanin extracted from the dried cells with a methanol solution containing 1% HCl was measured at 525 nm according to the method of Zhong et al. [10].

The HPLC analysis of triterpenes in the cultured cells was performed principally following the procedure we developed very recently [3]. Briefly, dried samples (0.5 g) were mashed

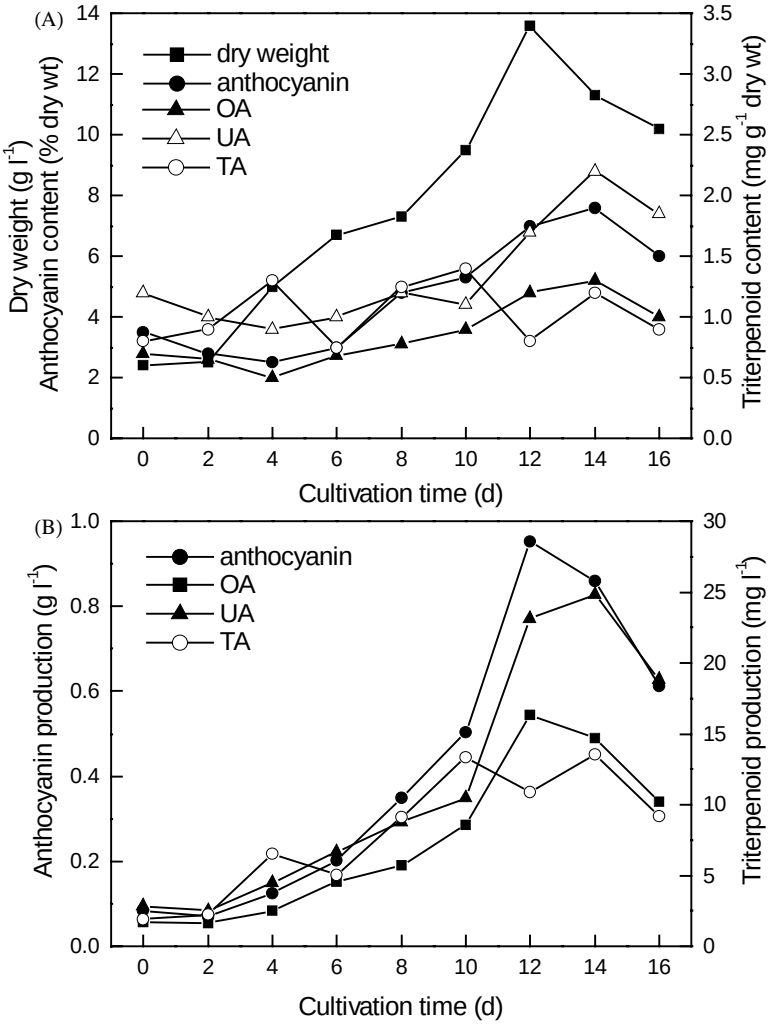

Fig. 3. Time course for cell growth, anthocyanin and triterpenoid accumulation in *Perilla frustescens* cell cultures. (A) Content of intracellular anthocyanin and triterpenoids, (B) total production of anthocyanin and triterpenoids in cultures. Anthocyanin or triterpenoid production refers to sum of the substance recovered from cells cultured in 1000 ml liquid medium. It was calculated by the formula: anthocyanin or triterpenoids content × cell dry weight. Values are means of triplicate results. Symbols: TA, tormentic acid; OA, oleanolic acid and UA, ursolic acid.

and refluxed thrice with methanol (50 ml, each) at 80 °C for 2 h. The extracts were combined and filtered while hot. Evaporation of solvent from the filtrate gave a residue which was dissolved in 10 ml of methanol. The afforded solution was filtered through a 0.45-μm syringe filter prior to the subsequent HPLC determination accomplished on a Waters 600E intelligent pump system equipped with a Waters 966 photodiode array detector with the detection wavelength set at 206 nm. The extraction solution was separated and analysed by using a 250 mm × 4.6 mm Spherisob ODS (5 μm) column at 25 °C. The mobile phase consisted of solvent A (acetonitrile), solvent B (water) and solvent C (4.4% H_3PO_4 aqueous, v/v). The following gradient procedure was used: 0–24 min, A:B:C = 50:48:2 (v/v); 24–43 min, A:B:C = 98:0:2 (v/v); 43–50 min, A:B:C = 50:48:2 (v/v). The flow rate was kept at 0.5 ml min^{-1}.

3. Results

3.1. HPLC profile of the methanol extracts from cell cultures

Three triterpenes such as TA, OA and UA previously found in leaves of *P. frutescens* [3] were successfully separated from the methanol extracts of cultured cells and analysed in a single HPLC run. Fig. 2A presented a typical chromatogram, which showed the separation of those three phytochemicals and their retention times acquired under the condition. Peaks 1–3 were identified as TA, OA and UA, respectively. Among the three triterpene acids, the content of TA (1.4 mg g^{-1} dry weight) was higher than those of UA (1.1 mg g^{-1} dry weight) and OA (0.9 mg g^{-1} dry weight).

3.2. Time course of cell growth and secondary metabolite formation

Data regarding the accumulation of biomass, anthocyanin and the triterpenoids are presented in Fig. 3. After 12 days of cultivation, the biomass was about 13.6 g dry wt l^{-1} which was about 5.7-fold of the initial concentration. The production of anthocyanin increased linearly with time up to day 12 with the highest value of about 0.9 g l^{-1} (7% of dry weight). As shown in Fig. 2A, the contents of OA and UA firstly decreased with time, then increased gradually until day 14 (1.3 and 2.2 mg g^{-1} of dry weight, respectively). The content of TA fluctuated between 0.8 and 1.4 mg g^{-1} of dry weight with the time course profile. Fig. 2B shows the time course for the production of both anthocyanin and the three triterpene acids in cell cultures. The highest production of OA, UA and TA (16.3, 24.9 and 13.6 mg l^{-1}, respectively) was achieved from day 12 to day 14.

3.3. Effect of yeast elicitor

As shown in Fig. 4, addition of yeast elicitor at all four given concentrations resulted in a decrease from 10.2 to 8.5 g dry wt l^{-1} in biomass accumulation. However, the elicitor treatment stimulated the accumulation of some secondary metabolites such as anthocyanin, OA and UA in cell cultures. Anthocyanin content peaked at 10.2% dry weight with yeast elicitor at 1% (v/v). Again evidenced from Fig. 4, the application of yeast elicitor reduced the TA production in all cases whereas the formation of OA and UA was enhanced by the elicitation at all concentrations. The chromatogram (Fig. 2B) also demonstrated an increased amount of OA (peak 2) and UA (peak 3) existing in elicited cells.

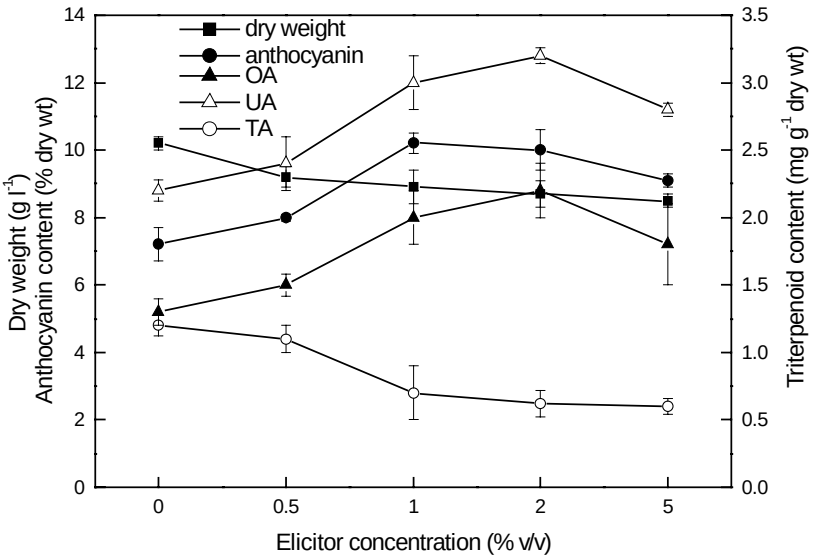

Fig. 4. Effects of yeast elicitor concentration on cell growth, anthocyanin and triterpenoid accumulation in *Perilla frustescens* cell cultures. Seven-day-old cell cultures were treated with yeast elicitor with different concentrations for 7 days. Control received 5 ml of distilled water only. Values are means of triplicate results and error bars show standard deviations. Symbols: TA, tormentic acid; OA, oleanolic acid and UA, ursolic acid.

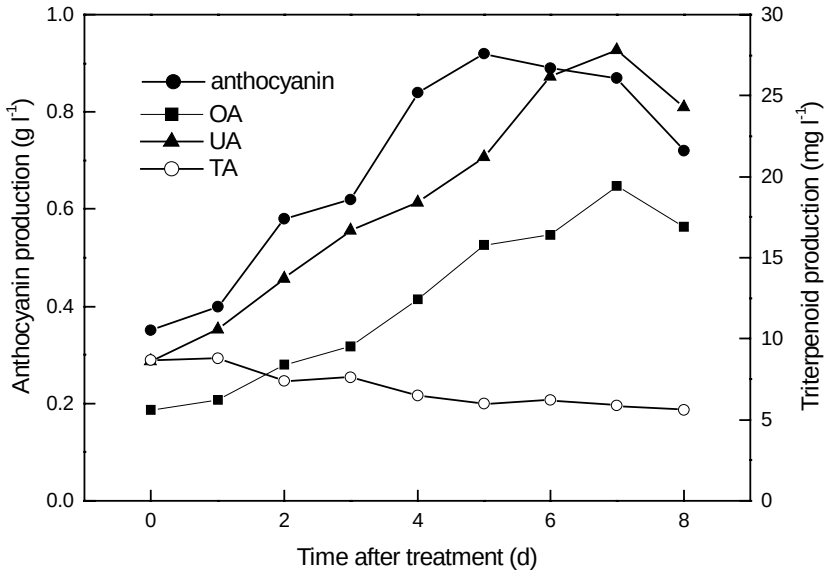

Fig. 5. Time course of the total production of anthocyanin and triterpenoids in *Perilla frustescens* cell cultures treated with yeast elicitor. The yeast elicitor at 2% (v/v) was added to 7-day-old cell cultures. Control received 5 ml of distilled water only. Anthocyanin or triterpenoid production refers to sum of the substance recovered from cells cultured in 1000 ml liquid medium. It was calculated by the formula: anthocyanin or triterpenoids content × cell dry weight. Values are means of triplicate results. Symbols: TA, tormentic acid; OA, oleanolic acid and UA, ursolic acid.

After 7 days of treatment with yeast elicitor at 2% (v/v), the content of OA and UA in elicited cells reached the maximum value (2.2 and 3.2 mg g^{-1}, respectively) as compared with that of the non-elicited control (1.3 and 2.2 mg g^{-1}, respectively). Therefore, the maximum production of OA and UA in the elicited cultures was 19 and 27 mg l^{-1}, a 46 and 24% increase over the control, respectively (Fig. 5).

4. Discussion

While producing a large amount of anthocyanin, the cell cultures of *P. frutescens* described here can synthesise as well the three pharmacologically important triterpene acids with the corresponding productivities comparable to those higher levels of the constituents (OA 0.07–0.18, UA 0.19–0.47 and TA 0.07–0.15% dry weight) in leaves of *P. frutescens* growing in different locations of China [3]. Owing to the conspicuous pharmacological activities of the three triterpene acids, biotechnological production of these substances using *P. frutescens* cell cultures is of great practical value.

Some pentacyclic triterpenoids such as OA and UA are suggested to be selective phytotoxins [11]. The present results show that accumulations of such allelochemicals can be stimulated by the applied yeast elicitor. And the maximum production of OA and UA in the elicited cultures was demonstrated to be 19 and 27 mg l^{-1}, a 46 and 24% increase over the control. This is the first report of a simultaneous production of both anthocyanin and triterpenoids in a single culture system of *P. frutescens* treated with a yeast elicitor. With strain improvement and optimisation of elicitation (such as elicitor purification, the incubation time for the elicitor treatment and physiological stages of cell aggregates), the greatly enhanced production of both anthocyanin and the triterpenoids in cell suspension cultures of *P. frutescens* could be expected.

Acknowledgments

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