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Short communication

Improved accumulation of phenylethanoid glycosides by precursor feeding to suspension culture of *Cistanche salsa*

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Abstract

Effects of some precursors on phenylethanoid glycosides (PeGs) accumulation in *Cistanche salsa* cell suspension cultures were investigated. Precursors such as tyrosine, phenylalanine, caffeic acid and cucumber juice at proper concentrations could increase the total accumulation of PeGs (echinacoside, acteoside, 2′-acetylacteoside) by 50%, 12%, 12% and 23%, respectively. Under the combined feeding of precursors at proper concentrations, the total production of PeGs in bio-staged culture reached the highest amount of 1358.1 mg l^{-1} (640.8 mg echinacoside l^{-1}, 689.4 mg acteoside l^{-1} and 54.9 mg 2′-acetylacteoside l^{-1}), which was about two-fold of that in the control. This study showed promise for obtaining large-scale production of active ingredients in plant cells by the solid–liquid two step culture (SLTSC) technique and also provided for the first time an example for producing PeGs by *C. salsa* cell culture. The improved production of PeGs was higher than that in previous reports on PeG production by *Cistanche deserticola* cell culture fed with precursors.

Keywords: *Cistanche salsa*; Phenylethanoid glycosides; Plant cell culture; Biosynthesis; Large scale cultivation; Precursor

1. Introduction

Phenylethanoid glycosides (PeGs), such as echinacoside, acteoside, 2′-acetylacteoside, and cistanoside, are the main pharmaceutically active components [1] in *Cistanche*, which is a traditional Chinese herb that grows in desert areas. They have marked activities for memory enhancement [2], sexual potency improvement [3] and free radical scavenging. They are also effective in anti-aging [4–7] and neuroprotection [8,9]. However, as *Cistanche* resources have become scarce [10], the recovery of secondary metabolites from plant cell cultures has been considered as an attractive alternative [11]. In plant cell culture, precursor feeding or elicitor treatment is an effective method to improve the accumulation of secondary metabolites in plant cell culture [12–15].

Molecules of PeGs consist of three parts: caffeic acid, aglycone and glucose (or rhamnose) [16]. Caffeic acid is a critical compound for the PeGs biosynthetic pathway. The pathway involves the metabolism of phenylalanine, which is converted by an ammonia lyase into *trans*-cinnamic acid, followed by hydroxylation at the 4-position of the aromatic ring forming 4-hydroxycinnamic acid or *p*-coumaric acid. Tyrosine can also be directly transformed into *p*-coumaric acid by an ammonia lyase. Further hydroxylation at the 3-position of the aromatic ring yields caffeic acid, which is subject to subsequent glycosylation resulting in PeGs [17–19]. The biosynthetic pathway reveals that caffeic acid, tyrosine and phenylalanine may serve as precursors for phenylethanoid glycoside synthesis.

Compared with the wild plant of *Cistanche salsa* and suspension culture of *Cistanche deserticola*, higher biomass and content of PeGs were obtained in the suspension culture of *C. salsa* in our previous study [20,21]. There are few researches on the production of PeGs in *C. salsa* cell suspension culture. In the present study, the effect of precursor feeding on the biosynthesis of PeGs in the cell suspension culture of *C. salsa* by using the SLTSC (solid–liquid two step culture) technique [22] were reported for the first time.

2. Materials and methods

2.1. Cell line and media

The CS2001 cell line was induced from *C. salsa* petals and subcultured in our laboratory. The growth medium was

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modified solid MS medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose, 2.0 mg l^{-1} 3-indoleacetic acid (IAA), 1.0 mg l^{-1} 6-benzyladenine (BA) and 2.0 mg l^{-1} Phytagel Gellan gum. The biosynthesis medium was modified liquid MS medium supplemented with 4% (w/v) glucose and 3.0 mg l^{-1} IAA. Various precursors were added into this medium.

2.2. Culture procedures

Callus cultures were incubated on the growth solid medium at 25 ± 2 °C in the dark. When the cells entered the fast growth period, 5.0 ± 0.2 g fresh cells were inoculated into 50 ml biosynthesis liquid medium held in 250 ml Erlenmeger flask and cultured in the dark for 4 weeks on a rotary shaker at 100 rpm. All experiments repeated three times and all values were the means of triplicate.

2.3. Precursor feeding

The preliminary experiment was designed to study the change of PeGs accumulation with the culture time. The experimental procedure was as described above. Accumulation of PeGs was monitored at 2-day interval for a period of 20 culture days. All of these were to forecast the optimal harvesting time of the suspended culture in the following experiments.

For experiments of precursor feeding, precursors with different concentrations shown in Tables 1–4 were added into suspension cultures of *C. salsa*. Cucumber juice, which contains much of caffeic acid, was obtained by centrifuging broken fresh fruit at 4000 rpm for 30 min at 4 °C. Considering the high cost of caffeic acid, phenylalanine, tyrosine, and cucumber juice were chosen as a combined precursor. The orthogonal design of this experiment is shown in Table 5.

Table 1
Effect of additional Phe on intracellular accumulation of the three phenylethanoid glycosides

Phe (mg l^{-1})	Echinacoside (mg l^{-1})	Acteoside (mg l^{-1})	2′-Acetylacteoside (mg l^{-1})	Sum (mg l^{-1})
Control 0	214.2 a	247.5 a	40.5 a	502.2
Phe 50	225.9 a	281.7 b	40.5 a	548.1
Phe 100	236.0 a[a]	284.0 b	44.0 a	564.0
Phe 200	196.3 b	293.6 b	40.6 a	530.5
Phe 400	121.8 b	259.0 a	31.7 b	412.5

Data are means of triplicate replication.
[a] Means ($n = 5$) in the column with different letters differ significantly ($P = 0.05$) according to Duncan's multiple range test.

Table 2
Effect of additional Tyr on intracellular accumulation of the three phenylethanoid glycosides

Tyr (mg l^{-1})	Echinacoside (mg l^{-1})	Acteoside (mg l^{-1})	2′-Acetylacteoside (mg l^{-1})	Sum (mg l^{-1})
Control 0	214.2 a	247.5 a	40.5 a	502.2
Tyr 100	248.4 a	236.7 a	41.4 a	526.5
Tyr 200	353.6 b[a]	356.2 b	41.6 a	751.4
Tyr 400	327.2 b	382.4 b	38.4 a	748.0
Tyr 500	251.5 a	322.0 c	42.9 b	616.4

Data are means of triplicate replication.
[a] Means ($n = 5$) in the column with different letters differ significantly ($P = 0.05$) according to Duncan's multiple range test.

2.4. Analytical method

The fresh weight of cells was taken after removing the excess moisture using filter paper and dry cells were obtained by freeze-drying until a constant weight was achieved.

Recovery of PeGs from the harvested dry cells was carried by extraction of 60% (v/v) methanol aqueous solution

Table 3
Effect of additional caffeic acid on intracellular accumulation of the three phenylethanoid glycosides

Caffeic acid (mg l^{-1})	Echinacoside (mg l^{-1})	Acteoside (mg l^{-1})	2′-Acetylacteoside (mg l^{-1})	Sum (mg l^{-1})
Control 0	386.1 a[a]	340.1 a	20.6 a	746.8
10	386.7 a	365.8 a	21.9 a	774.3
20	366.3 a	398.9 b	25.9 b	791.1
40	427.7 b	381.1 b	31.1 c	839.9
60	408.1 b	387.1 b	19.6 a	814.8
80	352.0 c	311.0 c	18.6 a	681.6

Data are means of triplicate replication.
[a] Means ($n = 3$) in the column with different letters differ significantly ($P = 0.05$) according to Duncan's multiple range test.

Table 4
Effect of additional cucumber juice on intracellular accumulation of the three phenylethanoid glycosides

Cucumber juice (ml l^{-1})	Echinacoside (mg l^{-1})	Acteoside (mg l^{-1})	2′-Acetylacteoside (mg l^{-1})	Sum (mg l^{-1})
Control	400.7 a	329.6 a	10.5 a	740.8
50	420.5 a	363.6 b	13.2 b	797.3
100	450.1 b	389.5 bc	11.2 a	850.8
200	489.4 b[a]	410.8 c	10.6 a	910.8
400	386.4 c	300.1 a	12.8 b	699.3

Data are means of triplicate replication.
[a] Means ($n = 3$) in the column with different letters differ significantly ($P = 0.05$) according to Duncan's multiple range test.