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Research Article

Establishment of Suspension Cell Culture from *Agrobacterium*-transformed Hairy Root Cells of *Psammosilene tunicoides,* an Endangered and Rare Medicinal Plant of China

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Abstract: *Psammosilene tunicoides* is an important medicinal plant endemic in China. Its annual yield is severely limited due to slow growth, poor seed germination and excessive collection. To satisfy the growing market demands, it's necessary to seek alternatives to field cultivation and wild resources of this endangered plant. Using *Agrobacterium*-transformed hairy roots as initial explants, here, we reported the development of a suspension cell culture system for *P. tunicoides*. Results showed the *Agrobacterium*-transformed hairy roots-derived suspension cells are fast in growth and strong in capacity for accumulation of bioactive metabolites. We established that 1/2MS was a suitable medium for culturing the hairy root-derived suspension cells and the optimal combination of phytohormones is 1.5 mg/L 2, 4-D+0.5 mg/L 6-BA+0.25 mg/L NAA+0.1 mg/L KT. Under this condition, the maximal biomass was achieved at the 20th day of culture with an average growth rate of 0.72 g/L/d; and the intracellular saponine content reached 0.92%, comparable to that of mother hairy roots. Compared with the normal *P. tunicoides* suspension cells, the hairy roots-derived suspension cells exhibited features of fast growth, short culture period and high concentration of saponines, suggesting that the large scale culture of hairy root-derived cells could be a feasible alternative to the wild resources of *P. tunicoides.*

Keywords: Hairy roots, *Psammosilene tunicoides*, suspension cells culture

INTRODUCTION

Psammosilene tunicoides W. C. Wu et C. Y. Wu is a perennial medicinal herb in the family of *Caryophyllaceae*. Its tuberous root has been widely used in traditional Chinese medicines to eliminate rheumatism, relieve pain and expel stasis (Deng *et al*., 2009). The bioactive components of *P. tunicoides* have been reported to be triterpenoid saponins, organic acids, cyclic peptides, lactam and modified amino acids, respectively. Recent studies also demonstrated the total of these components in this plant is much more effective, indicating that commercial supply of the *P. tunicoides* herbs heavily depends on over-collection of the wild plants (Zhang *et al*., 2012). However, the wild habitat of *P. tunicoides* has been severely destructed and its long growth period has drastically decreased the wild population of *P. tunicoides.* Consequently, it is necessary to seek alternative to field cultivation and wild resources to meet the increasing market demanding (Wang *et al*., 2002).

Large-scale culture of plant suspension cells, which is independent of environmental conditions and free of toxic compounds, has been suggested as promising alternative of conventional agriculture to obtain useful plant products. However, transition from laboratory-scale production of suspension cells to commercial-scale processes is technically challenging in terms of issues related with production titers and overall yields, including the low yield of plant metabolites, unstable producing ability of cultured cells and the slow growth rate (Mora-Pale *et al*., 2014). Hence, selection of desirable cells with suitable genetic, biochemical and physiological characteristics becomes the key techniques to solve the above-mentioned problems (Zhong, 2001).

Hairy roots, obtained by transforming plant cells with *Agrobacterium rhizogenes*, are clonal root cell lines which have been recognized as a viable constitutive and inducible plant bioproduction system for specialized phytochemicals (Guillon *et al*., 2006). Hairy roots induced by *A. rhizogenes* exhibit a number of advantages comparable to native plant roots, including fast growth in hormone free media, strong root branching, genetic and biosynthetic stability, plagiogeotropism and biosynthetic capacity (Srivastava and Srivasrava, 2007). Our previous studies have demonstrated that the hairy roots of *P. tunicoides*

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display identical saponines profile as the wild type roots and accumulate at the same or even higher level of phytochemicals than undifferentiated cells cultures and non-transgenic root cultures (Liu *et al*., 2011). However, one challenge to scale up these hairy roots is the clumping of hairy root tissues during continuous suspension culturing. The clumping leads the inner part of hairy roots to aging or even death because of long-term shortage of oxygen and nutrients and thus, further cause's severe declines in growth rate and yield of hairy roots (Sivakumar *et al*., 2011). Suspension culturing of cells established through callus which are induced from hairy roots will be a suitable alternative technology to overcome these problems, given its merits of good dispersion, stable genetics and fast growth rate of parental hairy roots. Recently, we have reported the success of induction and establishment of liquid culture system of *P. tunicoides* hairy roots (Li *et al*., 2011). Here, we further developed a suspension culture system from the hairy roots-derived callus. Comparing with the normal suspension cells, we showed that the hairy roots-derived cell culturing is a promising approach to produce suspension cells of *P. tunicoides* on industrial scale.

MATERIALS AND METHODS

Induction of *P. tunicoides* hairy roots: Leaves of sterilized seedlings were infected with *Agrobacterium rhizogenes* ACCC10060 for initiation of hairy roots and the hairy roots were cultured and maintained on solid MS supplemented with 2% sucrose + 0.45% agar as previously described (Li *et al*., 2011).

Induction and maintenance of callus from hairy roots: Tips of fresh *P. tunicoides* hairy roots were cut into 2~3 cm segments and placed on the MS supplemented with 0.5 mg/L 2, 4-D + 0.5 mg/L 6-BA. After culturing for 20 days under 25±1°C with darkness, formation of callus on the surface of roots was observed and this kind of callus was named as hairy roots-derived callus. As a control, the normal callus (derived from the normal leaves of *P. tunicoides* seedlings) was initiated and subcultured using the procedure as previously described (Wei *et al*., 2010). Both the hairy root-derived callus and normal leaf-derived callus were subcultured for 5 times and the fast-growing friable callus was chosen to further establish the suspension cell culture system.

Establishment of liquid culture system: The commonly-used basal media, MS, 1/2MS, B_5, 1/2B_5 and N_6, were selected for examining the suspension cell culture of *P. tunicoides* using the method of single factor test. Cell suspension cultures were initiated by inoculating 2 g (fresh weight) of callus (slightly crushed with sterilized tweezers) into a 250 mL Erlenmeyer flask containing 50 mL of liquid medium containing 3% (w/v) sucrose. Flasks were placed on an orbital shaker set with 110 rpm and 25±1°C in the dark. Subculture was conducted every 3 weeks until a homogenous cell suspension was achieved. Five replicates were included for each treatment.

Optimization of phytohormones in liquid media: Once the liquid basal media was selected, the orthogonal design was employed to examine the optimal combinations of various Plant Growth Regulators (PGRs). Two gram of fresh cells was inoculated into the preferred liquid media supplemented with various concentrations of 2, 4-D, 6-BA, NAA and KT either individually or in combinations as shown in Table 1 and 2 listed respectively and other culture conditions were the same as described in 1.3 unless otherwise specified.

Determination of optimal inoculums: Different amounts of initial cells (20, 40, 60, 80 and 100 g/L, respectively) were inoculated in the preferred basal media with optimal PGRs combinations and cultured under the same condition as above described. After 24 days of culturing, fresh suspension cells were harvested by centrifugation, treated through vacuum filtration, rinsed with distilled water for 3 times and weighed after removal of water by vacuum filtration (fresh weight, FW). When the fresh suspension cells were dried to a constant weight at 60°C in an electric oven, Dry Weight (DW) was weighed. The increase rate of biomass and growth rate were calculated as the following formulas:

$$\mu = (W_1\text{-}W_0)/W_0 \times 100\%$$
$$\nu = W_1\text{-}W_0/d$$

where,

μ : Increase rate of biomass
ν : Growth rate
W_0 : Initial inoculums
W_1 : Harvested weight
d : Days of culture

Determination of growth curve: Growth curve of suspension cells was examined under the conditions as

Table 1: Effects of PGRs on the suspension culture of normal *P. tunicoides* cells

Levels	A 2, 4-D (mg/L)	B 6-BA (mg/L)	C NAA (mg/L)	D KT (mg/L)
1	0.5	0.5	0.0	0.0
2	1.0	1.0	0.5	0.3
3	2.0	1.5	1.0	0.6
4	3.0	2.0	1.5	1.0

Table 2: Effects of PGRs for suspension culture of hairy root-derived cells

Levels	A' 2, 4-D (mg/L)	B' 6-BA (mg/L)	C' NAA (mg/L)	D' KT (mg/L)
1	0.5	0.00	0.00	1.0
2	1.0	0.25	0.25	0.1
3	1.5	0.50	0.50	0.2
4	2.0	1.00	1.00	0.3

described in above subsection. Suspension cells were collected every 4 days, washed with distilled water and the residual water was removed by filtration. DW was determined according to the procedure as previously published (Li *et al*., 2011) and the growth curve was plotted accordingly. Each treatment was repeated for 3 times with replicates.

Extraction and evaluation of total saponins: Extraction of total saponines in suspension culture cells was conducted according to methods previously described [10] and total saponins content was determined as previously published with modification (Zhang *et al*., 2013). Briefly, 5 μL of extraction solution was mixed with 0.5 mL vanillin solution (8%, dissolved with ethanol) and 5 mL H_2SO_4 (72%); the mixture was then incubated at 60°C for 10 min. After cooling to room temperature, the absorbance at 544 nm was measured. Calibration curves were established with oleanolic acid as the reference control.

RESULTS AND DISCUSSION

To evaluate the quality of hairy root-derived cells and to explore the feasibility of large scale culture of these cells, the normal suspension cell culture system was established simultaneously as control. Suspension cells initiated from hairy roots was named as Transformed Suspension Cells (TSC) and suspension cells from normal explants named as Normal Suspension Cells (NSC).

Growth response of TSC to basal media: Figure 1 showed the growth state of TSC and NSC on different basal media. The growth responses of TSC, measured as DW biomass accumulation and growth behavior, were tested on basal media MS, 1/2MS, N_6, B_5 and $1/2B_5$, respectively. Results showed that growth behavior of TSC and NSC performed differently in the same liquid basal media (Fig. 1). When cultured in liquid MS, NSC grew vigorously and dispersed homogeneously compared with those cultured in other four media. After 14 days, however, they became clumps of aggregate cells (diameter in size ranging from 0.8 to 3 mm) (Fig. 1a, b) and some adventitious roots emerged from the aggregates of NSC (Fig. 1c, d and e). Also, brown aggregates of suspension cells were observed in the ½ B_5 media (Fig. 1f). In liquid N6, the aggregates appeared mostly larger than 3 mm and gradually turning brown over the culture time (Fig. 1h). Generally, cultures in MS media exhibited comparatively less browning than those in other tested media. In subsequent experiments, MS was chosen as the basal media for the NSC culture. TSC cultured in MS, 1/2 B_5 or B_5, began to turn brown from the 7th day of culture. After 14 days, TSC in 1/2 MS grew well and had properties of homogenous cell particles and light yellow in color (Table 3). In contrast, TSC in other media became more and more brown and the browning degree can be ranked as $MS<1/2B_5<B_5<N_6$. Put together, 1/2 MS was chosen as the basal media for TSC in subsequent experiments.

Results showed the browning significantly influenced the suspension cell culture of *P. tunicoides* and this process appeared irreversible. Similar observation has been reported for the cell suspension cultures of *S. aemula* (Wang and Bhalla, 2006), presumably due to toxic compounds produced from the cultured cells. Given that the NH^{4+} content is low in the B_5 media and the nutrient composition much simple in N6, it is not surprising that neither B5 nor N6 could meet the requirement of *P. tunicoides* cell growth. In contrast, MS is high in inorganic metals and nitrate content, both essential for suspension cell culture of *P. tunicoides*.

Effects of PGRs on the TSC growth: It's recognized that different plant species requires different

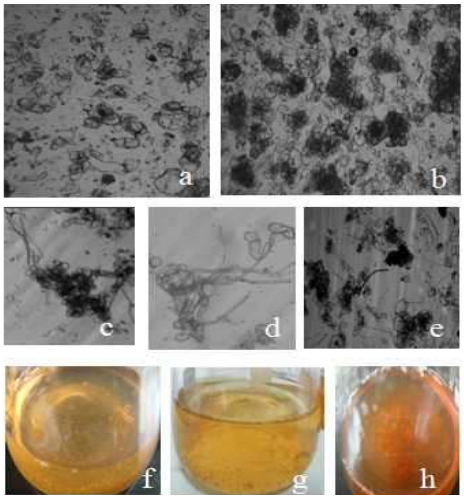

Fig. 1: Behaviors of suspension cultured cells (TSC) taken by bright field of inverted microscope motic AE31; (a): TSC on MS; (b): TSC on 1/2 MS; (c) and g Adventitious rooting from suspension cell aggregate and browning (TSC on B_5); (d): Adventitious roots from suspension cell aggregate (TSC on 1/2 B_5); (e): Rooting from suspension cell aggregates (TSC on N_6); (f): Typical photos for browning of suspension cells culture (TSC) taken by camera of Canon D-60; f Suspension cells cultured in 1/2 B5; (g): Suspension cells cultured in N6; (h): Suspension cells cultured in B5

Table 3: Browning of *P. tunicoides* suspension cell on different basal media

Basal media	Growth state	Degree of browning
MS	Browning	+
1/2MS	Normal	
B_5	Browning	++
$1/2B_5$	Browning	+
N_6	Browning	+++

phytohormones for cell division and growth. To select suitable growth regulators and optimize the concentration, we examined effects of PGRs on proliferation of TSC with orthogonal design (Table 1, 2 and 4). Results showed that different phytohormones have different effects on the growth suspension cells. For NSC, the order of effectiveness was 6-BA>2, 4-D>NAA>KT, with the optimal combination of 1.0 mg/L 2, 4-D+1.0 mg/L 6-BA+0.5 mg/L NAA. For TSC, by contrast, it was 6-BA>2, 4-D>KT>NAA, with the optimal combination of 1.5 mg/L 2, 4-D + 0.5 mg/L 6-BA+0.25 mg/L NAA+0.1 mg/L KT.

Effects of initial inoculums density on the growth of TSC: It's been known that initiation of plant suspension requires an appropriate concentration of cell inoculum. Minimum inoculation density, below which the cell growth doesn't occur or is preceded by a long lag phase, has been proposed (Kanokaree and Doran, 1997). Studies have demonstrated that the minimum inoculum density heavily depends on the properties of cell line (Wang and Bhalla, 2006; Sakurai *et al*., 1996; Akalezi *et al*., 1999; Reis *et al*., 2011). In this report, we investigated the relationship between initial inoculum of TSC and its growth. As shown in Fig. 2, the growth rate initially increased with the increase of initial inoculums and started to drop when it was greater than 80 g/L. For TSC, the maximum increase rate was achieved with the inoculum of 60 g/L, while it was achieved for NSC with 20 g/L.

Growth curves of both NSC and TSC: With a purpose to establish large scale-up production of *P. tunicoides* in vitro, we further determined the growth curve of the hairy root derived suspension cells (Fig. 3). Although it grew slightly slowly than NSC for the first 8 days of inoculation, TSC grew much faster during the day 8th and day 20th, with a peak rate of 14.6 g/L at the 20th day of culture. Afterwards, the cell growth stopped and biomass decreased gradually. In contrast, the maximum growth rate (0.43 g/L/d) and yield of biomass (12.4 g/L) for NSC were both gained at the 24th day of culture. Taken together, 20 days and 24 days were used as the growth period of TSC and NSC, respectively.

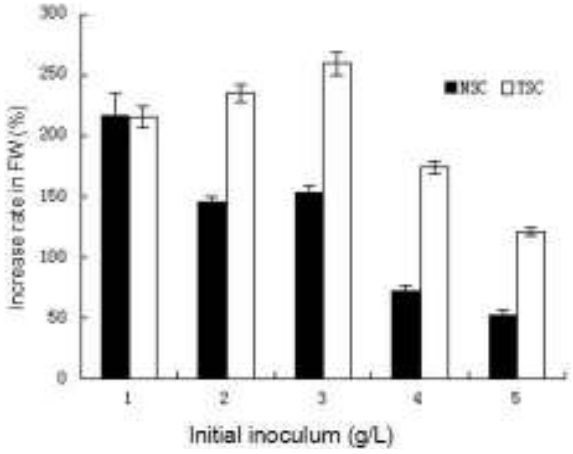

Fig. 2: Effects of inoculums density on the increase rate in fresh weight of suspension cells after 24 days of culture; (Line 1:20 g/L, line 2:40 g/L, line 3:60 g/L, line 4:80 g/L, line 5: 100 g/L)

Table 4: Results of orthogonal experiment

Factors	A (mg/L)	A'/B (mg/L)	B'/C (mg/L)	C'/D (mg/L)	D'(mg/L)	μ_1	μ_2
1	1	1	1	1	1	492.96	335.26
2	1	2	2	2	2	770.00	557.85
3	1	3	3	3	3	641.83	686.26
4	1	4	4	4	4	640.92	466.07
5	2	1	2	3	4	712.25	647.26
6	2	2	1	4	3	714.23	457.26
7	2	3	4	1	2	776.90	682.47
8	2	4	3	2	1	575.21	753.33
9	3	1	3	4	2	572.25	682.01
10	3	2	4	3	1	669.72	440.00
11	3	3	1	2	4	651.13	311.92
12	3	4	2	1	3	634.51	462.12
13	4	1	4	2	3	560.35	493.90
14	4	2	3	1	4	677.25	515.55
15	4	3	2	4	1	623.94	792.95
16	4	4	1	3	2	539.58	244.04
K_1	2545.71	2337.81	2397.90	2581.62	2361.83		
K_2	2778.59	2831.20	2740.70	2556.69	2658.73		
K_3	2527.61	2693.80	2466.54	2563.38	255092		
K_4	2401.12	2390.22	2647.89	2551.34	2681.55		
R_1	377.470	493.390	342.800	30.2800	319.720		
K_1'	2045.44	2158.43	1384.48	1995.40	2021.54		
K_2'	2540.32	1970.66	2160.18	2117.00	2166.37		
K_3'	1896.05	2173.60	2637.15	2017.56	2099.54		
K_4'	1746.44	1925.56	2082.44	2098.29	1940.80		
R_2	793.880	248.040	1288.67	121.600	225.570		

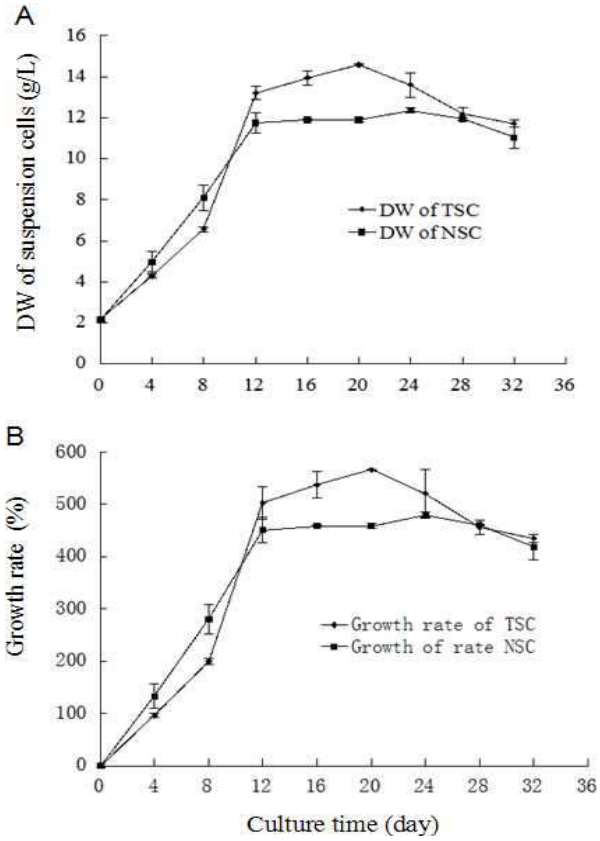

Fig. 3: Growth curves of two kinds of suspension cell culture

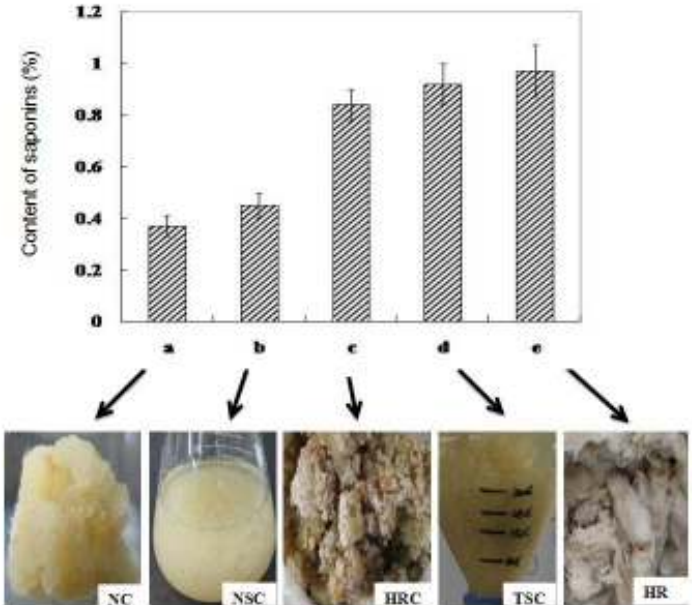

Fig. 4: Comparison of total saponins content between TSC and other cultures of *P. tunicoides*; (a) callus cultured on solid media, (b) NSC, (c) hairy root-derived callus cultured on solid media, (d) TSC, (e) hairy roots (HR: hairy roots; HRC: hairy roots-derived callus; NC: normal callus; TSC: transformed suspension cells; NSC: normal suspension cells)

Accumulation of total saponines in TSC: Hairy root, which is transformed by *Agrobacterium* species, are characterized with high growth rate, genetic stability and growth in hormone free media. More importantly, the level of secondary metabolites in hairy root can be comparable to that of intact plants (Srivastava and

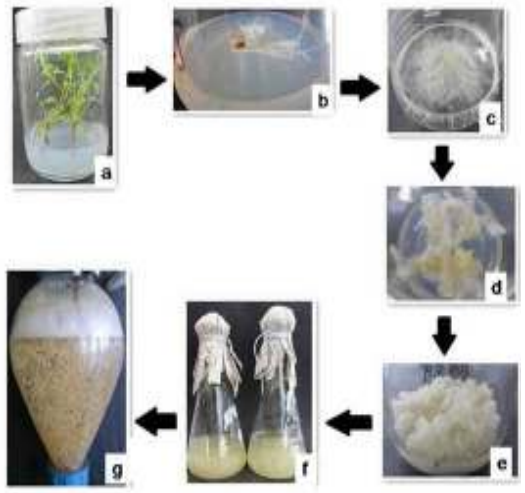

Fig. 5: Schematic diagram of suspension cells culture system initiated from hairy roots; (a) Aseptic seeding of *P. tunicoides*, (b) Hairy root induction, (c) Liquid culture of hairy roots, (d) Induction of hairy root-derived callus, (e) Hairy root-derived callus cultured on solid media, (f) Suspension cells cultured in a shaking-flask, (g) Suspension cells cultured in a 2.5 L micro-bubbling bioreactor

Srivasrava, 2007). Hence, it's conceivable that hairy roots-derived suspension cells remain the capacity of secondary metabolites biosynthesis and cellular proliferation of parental hairy roots. To examine the feasibility of large-scale culturing TSC for production of *P. tunicoides* bioactive compounds, total saponines content among hairy roots, hairy roots-derived callus, TSC, normal callus and NSC were compared. As shown in Fig. 4, both hairy roots of *P. tunicoides* and hairy roots-derived cultures including callus and suspension cells contained higher amount of total saponines (9.7±1.1 and 8.4±0.6 mg/g, respectively), in contrast to the normal callus and normal suspension cells (3.7±0.4, 4.5±0.5 mg/g DW, respectively). The content of saponines in TSC was comparable to that of mother hairy roots and slightly higher than that of hairy roots-derived callus inoculated on solid media. These results suggest it is feasible to explore the TSC of *P. tunicoides* for the production of active compounds with medicinal value in the liquid system.

Put these results together, we propose a procedure for *P. tunicoides* TSC culture system as 4 sequential steps:

- Induction and proliferation of hairy roots on solid media (Fig. 5a, b)
- Liquid culture of hairy roots (Fig. 5c)
- Induction and multiplication of hairy roots-derived callus on solid media (Fig. 5d and e)
- Suspension cells culture in flask and scale up of liquid culture (Fig. 5f and g)

At the early subcultures of suspension cells of TSC, it tends to form large aggregates of cells and could be removed from homogenous single or small particles of suspension cells by sieving under sterile conditions. After 5-8 times of subculture, stable and uniform TSC culture system can be established. An inspiring observation was that both biomass and yield of total saponines of TSC in 2.5 L bubbling bioreactor were comparable to that of mother hairy roots, suggesting that further scale up of TSC is potentially valuable.

CONCLUSION

The results presented in this study clearly indicated that hairy roots-derived Suspension Cells (TSC) culture system exhibited remarkable advantages compared to suspension cells from normal explants (NSC) in the respects of growth rate and secondary metabolites accumulation. First, TSC produces much higher levels of total saponines than that of NSC, comparable to that of the mother hairy roots. Second, fast growth of TSC facilitates to reduce the culture cost due to its short culture period. Third, utilization of TSC for production of secondary metabolites could avoid the problems existing in the process of liquid culture of hairy roots, such as clumping of hairy roots tissues, lack of uniformity of oxygen and nutrients transfer. In addition, growth media developed in this study will be useful in enhancing secondary metabolites accumulation and suspension cells proliferation. Different from hairy roots culture, which requires sophisticated equipments, the large scale culture of suspension cells is relatively simple in the design of bioreactors, suggesting the feasibility of the large-scale culturing hairy root-derived cells as a promising alternative to wild resources of *P. Tunicoides*.

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