INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
17–0251/2017/19–6–1535–1540
DOI: 10.17957/IJAB/15.0460
http://www.fspublishers.org

Full Length Article

Induction of *Psammosilene tunicoides* Adventitious Roots and the Accumulation of Triterpenoid Saponins as Affected by Culture Conditions

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Abstract

In this study, callus of *Psammosilene tunicoides* was used to induce adventitious roots. It was found that the medium with indole-3-butyric acid (IBA) was most suitable for induction of *P. tunicoides* adventitious roots from callus. To investigate the effects of concentration and type of auxins on adventitious roots induction, an orthogonal test was deployed. It was shown that 2,4-Dichlorophenoxyacetic acid (2,4-D) 0.3 mg/L+1-Naphthaleneacetic acid (NAA) 0.5 mg/L+ IBA 0.4 mg/L was the favorable auxins combination. We also established a suspension culture system in 500 mL flasks of *P. tunicoides* adventitious roots, and produced optimal culture conditions for metabolites accumulation with the sucrose concentration of 30 g/L, photoperiod at 10 h/d, and initial pH at 5.8, and IBA concentration at 0.4 mg/L. The resulting analysis highlights the results' useful in facilitating *P. tunicoides* rich in triterpenoid saponons for large scale cultivation. © 2017 Friends Science Publishers

Keywords: *Psammosilene tunicoides*; Auxin; Callus; Adventitious roots; Triterpenoid saponins

Introduction

On scale culture of plant cells or organs *in vitro* has been suggested as a promising alternative of field cultivation to achieve natural compounds, especially to those endangered and rare plant species with high medicinal value (Jeong *et al*., 2009). For example, ginsenosides production can be enhanced through the use of bioreactor based systems (Yu *et al*., 2000; Kim *et al*., 2004) and the liquid culture of *Echinacea purpurea* adventitious roots could lead to higher accumulation of caffeic acid derivatives than of intact plants (Wu *et al*., 2007). However, large-scale cultivation of plant suspension cells requires optimization of physical and chemical conditions, which vary according to the following: plant species, culture tissues, and culture objectives.

These research efforts concentrate on the induction of adventitious roots from the callus of *Psammosilene tunicoides*, which is a perennial herbaceous plant in the family *Caryophyllaceae*, native to South-western China. In the traditional Chinese medicine, the tuberous roots of *P. tunicoides* have been widely utilized to diminish rheumatism, alleviate pain and discharge stasis (Deng *et al*., 2009). Recent studies demonstrate that the roots of this plant are also effective to improve the immune system, alleviate skin swelling, and possess significant analgesic effect for rheumatic arthralgia (Zhao *et al*., 2011). Biochemical analyses have shown the bioactive components of *P. tunicoides* are could be any of the following: cyclic peptides, triterpenoid saponins, organic acids, lactam or modified amino acids (Zhao *et al*., 2011).

In this study, we aimed to develop a proof of concept method for producing the high yield of saponins from adventitious roots cultures incited from *P. tunicoides* are callus. We tested various physical-chemical parameters, reported the information on auxins types and concentrations for induction of adventitious roots, and optimized culture conditions for the accumulation of biomass and saponins yield from *P. tunicoides* adventitious root cultures.

Materials and Methods

Materials

Psammosilene tunicoides callus were provided by the Laboratory of Medicinal Plant Cell Engineering of Dalian Polytechnic University. Initially callus was induced from sterile seedlings and cultivated on solid MS medium, which was supplemented with 3% sucrose, 4 g/L agar powder, with pH adjusted to 5.8 and sterilization under the condition of 121ºC, 1.2kg/cm^2, 15 min.

For maintenance, the callus was cut into 0.5 cm × 0.5 cm pieces, and inoculated on solid MS medium, kept at 25±1ºC with photoperiod 12 h/d and light intensity 2000 Lx.

To cite this paper: Zhang, Z., D. Zou, M. Liu, M. Cong, N. Cheng, H. Gao, S. Wang, Z. Liu and G. Li, 2017. Induction of *Psammosilene tunicoides* adventitious roots and the accumulation of triterpenoid saponins as affected by culture conditions. *Int. J. Agric. Biol*., 19: 1535–1540

Combinations and Concentrations of Auxins on the Induction of Adventitious Roots

Indicated auxins were supplemented to culture medium at the following concentrations, respectively: NAA (0, 1.0, 2.0 and 3.0 mg/L), 2, 4-D (0, 0.5, 1.0 and 1.5 mg/L) and IBA (0, 0.5, 1.0 and 1.5 mg/L). Orthogonal test was adopted to investigate the effect of three types of auxins (2, 4-D, NAA and IBA) at different concentrations on the induction of *P. tunicoides* adventitious roots (Table 1).

Optimization of Culture Conditions of Adventitious Roots

The adventitious roots were cut into 1 cm length after cultured for 20 days, and transferred into 3 L bioreactor containing 2.1 L liquid MS medium, with an inoculum size of 5 g/L. The bioreactor was aerated at a speed of 160 cc/min controlled by air flow meter.

The sucrose concentration, initial pH, IBA concentration and photoperiod were investigated to examine the growth of *P. tunicoides* adventitious roots. The culture conditions were set as below: sucrose concentration: 0, 20, 30, 40 and 50 g/L; auxins concentrations: NAA (0.5, 1.0 and 1.5 mg/L), IBA (0.1, 0.2, 0.3, 0.4 and 0.5 mg/L); initial pH was set at 5.0, 5.5, 5.8, 6.0 and 6.5; photoperiod: 0, 8, 10, 12 h/d and the light intensity was 2000 Lx. For these experiments, the culture temperature was 25±1ºC, unless specified otherwise. After 30 days of cultivation, fresh and dry root weights, growth ratio and the content of triterpenoid saponins were measured.

Determination of Roots Weight and Growth Ratio

The efficiency of auxins for inducing adventitious roots from callus was measured in the following way.

Induction ratio = harvested numbers/inoculated numbers × 100%

Where harvested numbers mean the numbers of callus that induced adventitious roots; inoculated numbers are all of the inoculated callus numbers.

Relative Growth Ratio (RGR) was determined according to the formula:

$$RGR = t^{-1} \cdot \ln (W_2/W_1)$$

Where W_1 was the dry weight of inoculated roots; W_2 was the dry weight of harvested roots; *T* is the cultivation time. The above tests were repeated twice and averaged.

The fresh weight (FW) and dry weight (DW) of the roots were measured at the culmination of 4 weeks, as follows: roots were shifted from the medium by passing through a 16 mesh stainless steel sieve, with the FW measured after cleansing once with sterile water and blotting away the surface water; the DW was recorded after drying the roots to constant weight at 50ºC for 2 days (Zhang *et al*., 2013).

Table 1: Effects of exogenous auxins on the induction of adventitious roots from callus

Auxins	Concentration (mg/L)	Induction ratio (%)	Growth rate	Surface color of callus
2,4-D	0.5	29.2d ± 2.4	++	milky white
2,4-D	1.0	12.5e ± 4.2	++	brown
2,4-D	1.5	4.2e ± 0.0	+	light yellow
NAA	1.0	41.7c ± 4.2	+++	milky white
NAA	2.0	62.5b ± 12.5	++	grey white
NAA	3.0	33.3cd ± 4.2	++	light yellow
IBA	0.5	75a ± 8.3	+++	milky white
IBA	1.0	33.3cd ± 0.0	++	milky white
IBA	1.5	8.3e ± 4.2	++	light yellow

Note: growth rate: slow+, faster++, fastest+++

Determination of Growth Curve

Growth curve of suspension adventitious roots was observed under the conditions described above. Collection of the roots occurred every 3 days, washed with distilled water, with any residual removed by filtration. Each treatment was repeated for 3 times with replicates.

Determination of Content of Triterpenoid Saponins

Ground-dried roots (0.5 g) were suspended in 10 mL 80% ethanol ultrasonic for 100 min and then swung dry the extract by rotary evaporator. The extract was then dissolved with 10 mL deionized water, and re-occupied with N-butyl alcohol (4:3:3) for three times, then swung dry again. At last, the extract was dissolved with 10 mL methanol and the solution was used to determine the content of saponins.

Vanillin-perchloric acid colorimetric method was adopted to determine the content of total saponins in adventitious roots (Liu *et al*., 2011; Zhang *et al*., 2013). The steps were as follows: The prepared extracts (0.5 mL) were mixed with 0.2 mL 5% vanillin-perchloric acid and 0.8 mL perchloric acid solution, dried by water bathing at 65℃for 20 min. After cooling to the room temperature, the mixture was added with 5 mL glacial acetic acid, and the absorption level was measured at 540 nm by a UV visible spectrophotometer. The amount was compared to a standard curve prepared using R_e ginsenoside, and expressed as the mean, in mg, of R_e ginsenoside equivalent per gram of dried plant material from triplicate extracts.

Statistical Analysis

The results were formulated as the mean value of three independent experiments. For weight measurement, content of triterpenoid saponins, growth curve, a one-way analysis of variance (ANOVA) was applied to the data. Assessments of the statistical difference between the mean values were performed using a least difference (DUNCAN) test, with *P*-values< 0.05, using the SPSS program (IBM Institute, Inc., U.S.A.). In figures, the values were marked for their significance levels compared to the control.

Results

Effects of Types and Concentrations of Auxins on the Induction of Adventitious Roots

When the three auxins were respectively used alone in different concentrations to induce adventitious roots, the highest root induction ratio of 75% was observed with 0.5 mg/L IBA (Table 1). With this treatment, the induced roots grew with branches and the growth rate appeared faster compared to those treated with other auxin types. At higher IBA concentration, however, root induction ratio significantly decreased. Moreover, callus browning was observed with high concentration of IBA, possibly due to the accelerated aging of callus associated with the high concentration of IBA.

NAA was the second effective auxin to induce formation of adventitious roots. When NAA was added to culture medium with a concentration of 2.0 mg/L, the root induction rate was calculated as 63.5%. With the other growth regulators, the induction ratio appeared less than 30%. The types and concentrations of auxins affected the length, the color and the numbers of branches of adventitious roots were found. The results showed that the IBA was suitable for induction of adventitious roots from *P. tunicoides* callus.

After successful induction of adventitious roots from callus, they were separated and transferred to solid fresh media of MS from callus proliferation. And then the callus was used as materials for induction of adventitious roots (Fig. 1B). At presence of 0.5 mg/L IBA the formation of *P. tunicoides* adventitious roots was visible after 12 days of culture (Fig. 1C). After 24 days, a number of white adventitious roots could be observed as clusters on the callus surface (Fig. 1D).

After induction and proliferation of adventitious roots on solid MS, an appropriate initial inoculum of adventitious roots was collected and transferred into liquid MS for following experiments. The establishment of liquid culture system showed that it needs about 30 days from initial inoculation to maximum yield of biomass (Fig. 2).

Effect of Sucrose Concentration on the Growth of *P. Tunicoides* Adventitious Roots in Liquid Media

Sucrose concentration in culture medium produced a significant effect on adventitious root growth. The concentration of 30 g/L resulted in a maximum growth ratio of 14.2±0.4 (Fig. 3A). The results also indicated that sucrose concentrations higher than 30 g/L inhibited roots growth and reduced root biomass accumulation, possibly due to a higher osmotic potential of cultured cells/organs. However, the accumulation of total saponins was correlated with the increase of sucrose concentrations above 30, 40 and 50 g/L sucrose. The increases of initial sucrose concentration significantly increased the accumulation of total saponins

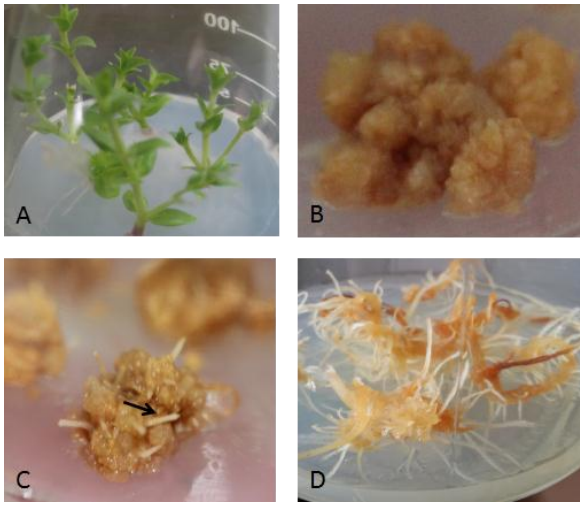

Fig. 1: Induction and growth of adventitious roots of *P. tunicoides* from the callus on the solid MS. (A: Sterile seedling; B: Callus derived from sterile seedling;C. Adventitious roots developed from the surface of callus; D: Multiplication of adventitious roots on solid MS)

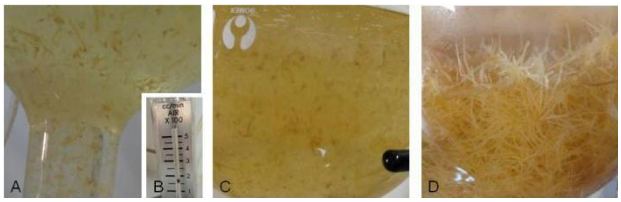

Fig. 2: Developed adventitious roots of *P. tunicoides* were separated and transferred into liquid MS for further culture (A. Initial inoculation; B. Air flowmeter indicates at 160 cc/min; C. Cultured in liquid MS for 15 days; D. Cultured in liquid MS for 30 days)

(Fig. 4A). It has been reported that for adventitious root cultures sugar supplement acted as a carbon source; and its initial concentration can affect several *in vitro* parameters, e.g., growth and the yield of secondary metabolites.

Effect of Photoperiod on the Growth of *P. Tunicoides* Adventitious Roots

With the extension of photoperiod, the growth ratio and saponins content showed a trend of increasing and then decreasing, and maximized when the photoperiod was 10 h/d. These results suggested that proper illumination can promote both the root growth and the accumulation of saponins (Fig. 3B and 4B).

Effect of Initial pH on the Growth of *P. Tunicoides* Adventitious Roots

The initial pH has a significant influence on the cell growth and saponins content of *P. tunicoides*

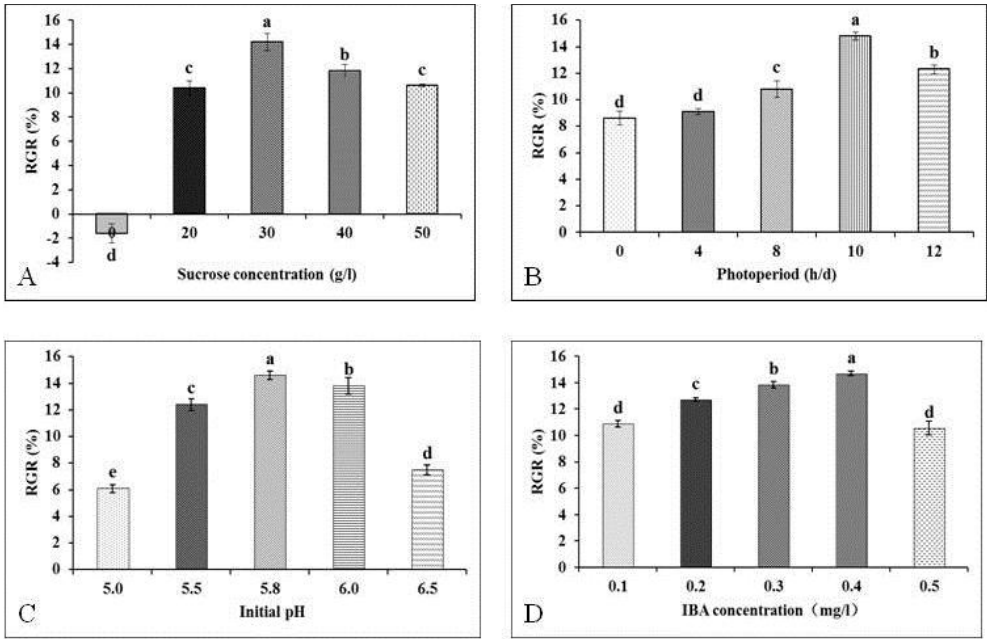

Fig. 3: Effects of four factors on the growth of *P. tunicoides* adventitious roots (*A. Sucrose* concentration; *B. Photoperiod*; *C. Initial* pH; D.IBA concentration)

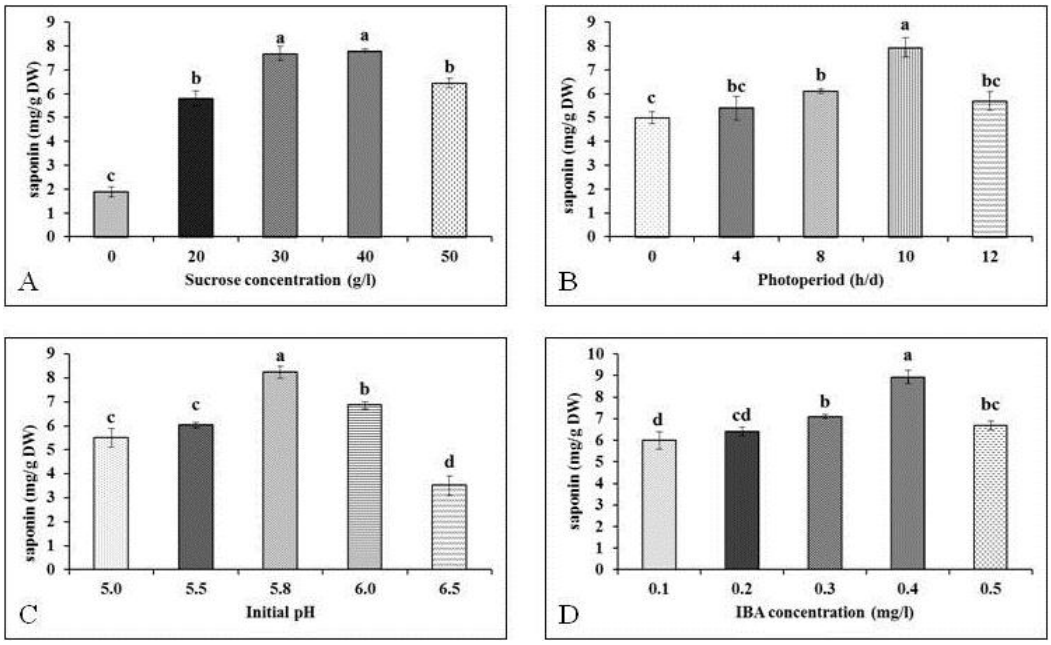

Fig. 4: Effects of four factors on the content of saponins of *P. tunicoides* adventitious (*A. Sucrose* concentration; *B. Photoperiod*; *C. Initial* pH; D.IBA concentration)

adventitious roots. With the changes of initial pH, the relative growth ratio and saponins content showed a trend of increasing and then decreasing, and the relative growth ratio maximized (14%) with the initial pH of 5.8. These results were in agreement with the photoperiod regulation (Fig. 3C and 4C).

Effect of Exogenous IBA on the Growth of *P. Tunicoides* Adventitious Roots

The impact of concentrations of exogenous IBA on the growth of adventitious roots under liquid conditions showed that with the increase of IBA concentration, the

relative growth ratio and the saponins content of adventitious roots increased initially, peaked at the concentration of IBA at 0.4 mg/L, and then decreased afterwards (Fig. 3D and 4D).

Growth Curve of *P. Tunicoides* Adventitious Roots

In order to optimize the subculturing conditions and harvest period, we investigated the growth curve of adventitious roots. As shown in Fig. 5, growth curve exhibited a standard "S"form. The lag-phase was approximate 6 days, and the biomass of adventitious roots was almost unchanged during this period. Roots started to proliferate at the 7th day post of inoculation when the exponential growth phase started. At the 25th day, the root proliferation rate started to decrease, and at about the 30th day, the growth curve started to flatten into quiescent, the proliferation rate and mortality got into equilibrium. These results suggested that *P. tunicoides* adventitious roots should be sub-cultured at about 20th d, while the harvest period can be 30 days to gain a higher biomass.

Discussion

Liquid culture of adventitious roots would be a potential alternative to field cultivation, even to suspension cell and tissue culture in the production of natural chemicals from medicinal plants, especially for those of rare and endangered plant species. According to the theory of cell totipotency, organs could be developed and obtained through dedifferentiation and re-differentiation of embryogenic cells with the regulation of phytohormones *in vitro*. Numbers of studies have demonstrated that different auxin has its own acting mechanism in inducing roots, however, the existence of the threshold concentration might be in common among those auxins, such as IAA, NAA, IBA, and 2, 4-D, *etc*. Besides, the combination of various auxins is another crucial factor regarding to roots induction in tissue culture.

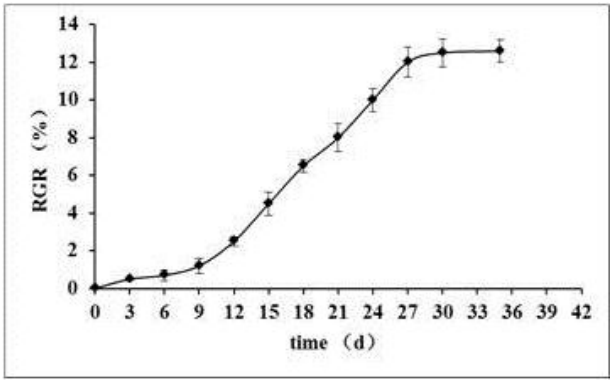

Fig. 5: Growth curve of *P. tunicoides* adventitious roots cultured in 500 ml flask. Growth curve exhibited a standard "S" form

In this report, we adopted the orthogonal test to investigate the effect of types and concentrations of auxins on the induction of *P. tunicoides* adventitious roots. In the case of present study, the callus of *P. tunicoides* was taken as initial explants to induce adventitious roots, and IBA was proven to be the most suitable for the induction of adventitious roots from callus, which conforms with the previous experiment result using other different plant species; for example, in *Panax notoginseng* and *Centaurea tchihatcheffii tchihatcheffii*, IBA was effective to induce adventitious roots (Hahn *et al*., 2003; Ozel *et al*., 2006). In addition, the favorable auxins combination is IBA (0.4 mg/L) + NAA (0.5 mg/L) + 2, 4-D (0.3 mg/L), and a higher induction rate of adventitious roots was realized in this case.

To establish liquid culture protocol of *P. tunicoides* adventitious roots, we also employed 500 mL flasks with liquid media to culture *P. tunicoides* adventitious roots after collection from callus, and then optimized the suitable culture condition for biomass accumulation. The preferred parameters for adventitious roots growth were of 10 h/d photoperiod, initial pH 5.8, and of 0.4 mg/L IBA. It is possible that the initial pH of medium can not only affect the efficiency of cells/organs to absorb carbon and nitrogen in culture system, but also can affect the accumulation and release of secondary metabolites and the enzyme activities of the cells/organs. Similarly, it was found that for *Echinacea angustifolia* adventitious roots, initial pH between 5.0 and 6.0 was the most favorable to the growth and accumulation of flavonoids and polyphenols (Wu *et al*., 2006; Rohela *et al.*, 2016). However, as to photoperiod, this result appeared different from the observations of *Echinacea purpurea* where adventitious roots cultured in dark (0h/d) accumulated higher biomass (Wu *et al*., 2007). This difference is most likely due to different sensitivity of *P. tunicoides* and *Echinacea* roots to light intensity and duration of photoperiod.

Conclusion

Taken together, these findings demonstrate that the growth period of different plants and different organs of one plant are different. These results are useful for large scale cultivation of adventitious roots of *P. tunicoides* which were rich in triterpenoid saponins, and thus provide the theory and practice basis for development of series of medicines and health products.

Acknowledgements

Authors thank the National Science Fund of China for financial support to this work (31070164), and also thank the Excellent Talents Fund Project of Henan Province for aiding this research in part (134200510002).

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(Received 04 March 2017; Accepted 15 August 2017)