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Research Article

Liquid Culture of Adventitious Roots is a Potential Alternative to Field Cultivation for *Psammosilene tunicoides*, a Rare and Endangered Endemic Medicinal Plant

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Abstract: The aim of this study was to establish an adventitious roots culture system for sterile plantlet segments of *P. tunicoides* and improved the accumulation of total saponins in cultured roots. *Psammosilene tunicoides* is a native Chinese plant with high commercial value as medicinal herb. Combination of NAA and IBA significantly affected the adventitious roots formation on agar-solided B_5 media and a maximal induction rate of 83% was obtained at 24±2°C with a photoperiod of 12 h. With a shaking of 110 rpm in darkness, transferring the detached adventitious roots to the growth regulator free 1/2 B_5 liquid media notably increased the biomass production compared to that on solid media over a 30-day-culture period. Further analyses showed that more saponins could be accumulated in the liquid culture than in the solid culture and the addition of exogenous oxalic acid to the liquid media could enhance the accumulation of total saponins in adventitious roots. These results suggested that adventitious roots culture will be an efficient alternative to the field cultivation of intact plants for the production of useful natural compounds from *P. tunicoides*.

Keywords: Adventitious roots, liquid culture, *Psammosilene tunicoides*, saponin

INTRODUCTION

Psammosilene tunicoides W. C. Wu et C. Y. Wu is a herb species in the *Psammosilene* genus of Caryophyllaceae. Endemic to the South-Western region of China, this plant is primarily distributed at an elevation of 2600-3200 m, in the crevices of rocks (Wang *et al*., 2002). Its root has been widely used as medicinal herbs in China for exhibiting various therapeutic effects, such as stopping bleeding, relieving pain and promoting blood circulation (Deng *et al*., 2009). Research results have identified the active components of *Psammosilene* as saponins and cyclic peptides. Crude saponins obtained from this plant have been shown to possess a strong activity of pain-relieving and anti-inflammatory (Wang *et al*., 2006). Over the past decade, market demand for *P. tunicoides* has dramatically increased in China and other Asian countries and *P. tunicoides* has been facing an imminent danger of extinction due to the habitat destruction and illegal collection. In face of the acute scarcity of the plant, China has enlisted this plant as an endangered and rare plant species, inevitably affecting its market supply in future. Hence, it's urgent and necessary to seek new means to reproduce this plant. Adventitious roots induced by in vitro methods showed high rates of proliferation and active secondary metabolism and thus offers a viable alternative for providing *P. tunicoides* in the pharmaceutical industry. Adventitious roots are of natural product, grow vigorously in phytohormone-free media and possess great potential to accumulate valuable secondary metabolites. Cultivation of adventitious roots has been suggested as an alternative for natural compounds production. In the field of plant biotechnology, hairy roots, induced by Ri plasmid insertion, has been reported to be a promising pathway for producing rare and endangered medicinal plant species (Zhong *et al*., 2002). However, compared with hairy root cultures and field cultivation, this approach is safer, more stable and easier for management (Sudha and Seeni, 2001). To our knowledge, however, culturing adventitious roots of P. tunicoides has not been reported yet. In this paper, we developed a liquid culture system for *P. tunicoides* adventitious roots and laid a foundation for large-scale production of secondary metabolites in *P. tunicoides* adventitious roots and could be expected to be an alternative to filed cultivation or wild resources.

MATERIALS AND METHODS

Plant material: Sterile plantlets of *P. tunicoides* were grown on agar-solidified B_5 medium supplemented with sucrose 25 g/L + 6-BA 0.05 mg/L + IAA 0.05 mg/L at

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a temperature of 22-24°C and illuminated by 1800 lux with a photoperiod of 12 h for 25 days.

Adventitious root cultures were induced from the sterile plantlets of *P. tunicoides* and sub cultured every 30 days by adding 0.1 g roots (about 10 pieces of roots) to a 100 mL flask containing 25 mL of B_5 liquid medium without phytohormone. Cultures were shaken using a rotary shaker at 110 rpm under 22±2°C in the dark.

Callus, from the sterile plantlets of *P. tunicoides*, was induced and maintained on the agar-solidified B_5 (sucrose 25 g/L + 6-BA 0.05 mg/L + IAA 0.05 mg/L) under the same culturing conditions with that of sterile plantlets but without light. And the cultures were sub-cultured every 30 days.

Elicitor treatment: The experiment of *in vivo* elicitation with oxalic acid was carried out according to the procedure described by Zhang *et al*. (2009) with modifications [19]. Oxalic acid was dissolved in distilled water. The pH of this solution was adjusted to 5.6 with NaOH and autoclaved for 30 min which was added to the medium at specified time under sterile condition. The elicitor was added at a final concentration of 10^{-5} mol/L in 0, 7, 15 and 20-day-old suspension cultures, respectively and incubated for a further period until the 30th day of culture. Control cultures were treated with sterile distilled water.

Extraction and estimation of total saponins: On the specified days post of culturing, the adventitious roots, together with callus and plantlets, were harvested and dried to a constant weight at 60°C in an electric oven and then ground into fine powder. Total saponins were extracted with 80% methanol and ultrasonicated for 1 h at 40°C. The extract was centrifuged at 1000 rpm for 10 min and evaporated to dryness. The dried pellet was re-extracted with water-saturated *n*-BuOH. After evaporating the *n*-BuOH phase phase to dryness, a weighed pellet was dissolved in water to some volume.

Total saponins content was determined according to the procedure previously published with subtle modification (Tian *et al*., 2009; Zhang and Wang, 2009). Five μL of extraction solution was mixed with 0.5 mL vanillin solution (8%, dissolved with ethanol) and 5 mL H_2SO_4(72%) and then the mixture was incubated at 60°C under water bath for 10 min, after rapid cooling to room temperature, the absorbance was measured at 544 nm. Calibration curve were established with ginsenoside *Re* as reference saponin.

Statistic analysis of data: The growth ratio was calculated using the following equation and recorded as means ±S.D. of data obtained from three experiments.

Growth ratio = $(W_2 - W_1) / W_1$, where W_1 is the dry weight of biomass in time t_1 (g) and W_2 represents the harvested dry weight of biomass (g) in time t_2.

Relative growth rate was calculated from dry weight and meant the average productivity per day and was from the formula of $(W_t - W_0) / (W_0 \cdot t)$, W_0 is the dry weight of the inoculums (g), W_t is the dry weight of biomass (g) at time t (d).

RESULTS AND DISCUSSION

Induction and culture of adventitious roots from segments of sterilized plantlets: We first tested the effects of plant growth regulators on the formation of adventitious roots on solid B_5. (2-3 cm) segments of sterilized plantlets were used as explants for initiation of adventitious roots. At the 9th-12th day of inoculation in dark, adventitious roots formation was observed form the cut end. As shown in Table 1, the highest frequency of adventitious root induction occurred in the combination of 0.05 mg IBA + 0.1 mg NAA, with a maximum of 83%, followed by combination of 0.1 mg IBA + 0.1 mg NAA (39%), 0.2 mg IBA + 0.1 mg NAA (9%) and 0.4 mg IBA + 0.1 mg NAA (3%). Different morphologies of the adventitious roots were also recorded between different treatments and they could be classified into three types:

- **Type 1:** Thin and short roots in the presence of IBA alone (Fig. 1 C)
- **Type 2:** Fast growing and thick roots in the presence of NAA alone (Fig. 1 B)
- **Type 3:** Fast growing and highly branched roots in high ratio of NAA to IBA relatively (Fig. 1 D)

In our study, we found that auxins involves the root formation process, in agreement with previous results that application of exogenously applied auxins would aid adventitious rooting, although this may not be a universal requirement across species (Staden and Harty, 1988; Cristofori *et al*., 2010). Clearly, our data showed that the type and concentration of auxins strongly influenced the formation of adventitious roots (Table 1, Fig. 1) and also further indicated that IBA is an effective inducer of adventitious root formation. Low ratio of IBA to NAA appeared more efficient to induce adventitious rooting.

Establishment of liquid culture system for adventitious roots of *P. tunicoides*: After induction and culturing on solid B5 for 14-17 days, the adventitious roots were detached from the explants aseptically and transferred into the liquid 1/2 B5 media

Table 1: Effects of IBA & NAA on the formation of adventitious roots of *P. tunicoides* cuttings

Combinations of IBA & NAA	Numbers of explants	Numbers of adventitious roots	Percentage of rooting (%)
0.05 IBA + 0.1 NAA	90	75±2.5	83
0.1 IBA + 0.1 NAA	90	39±1.3	43
0.2 IBA + 0.1 NAA	90	9±0.80	10
0.4 IBA + 0.1 NAA	90	3±0.60	3

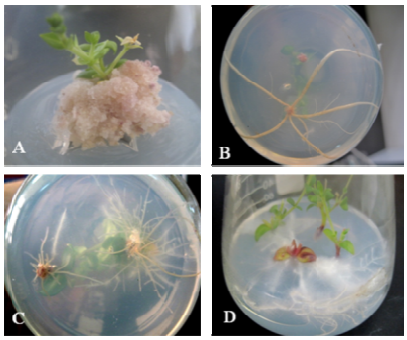

Fig. 1: The effects of IBA and NAA on the formation of adventitious roots at the segments base of *P. tunicoides* at 17 days (A) only formation of callus when the ratio of NAA/IBA was more than 4, (B) Less branch roots occurred at the main root in the presence of NAA alone, (C) Addition of IBA alone, thin and short roots in the presence of IBA alone, (D) In low ration of IBA to NAA, relatively highly branched roots compared with that treated with NAA in (B) and (C)

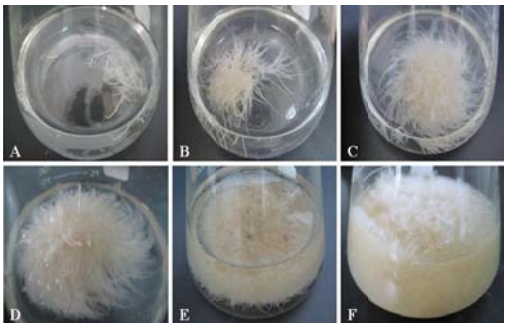

Fig. 2: Profiles of multiplication of adventitious roots in the liquid B5 showing high growth rate (A) Initiation stage, (B) 7 days, (C) 13 days, (D) 17 days, (E) 25 days, (F) 45 days

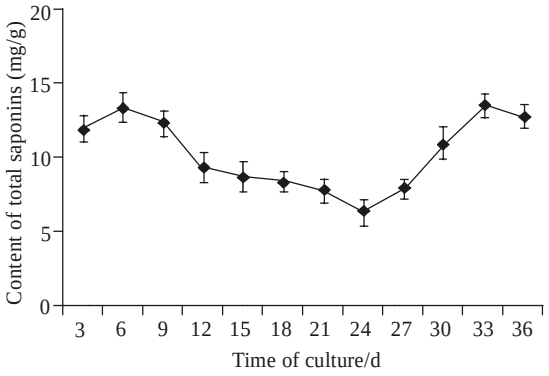

Fig. 3: Changes in total saponins concentrations during liquid culture of hairy roots with time

without phytohormone. When the initial inoculum was 0.624 g/100 mL, it reached a maximum of 8.250 g/100 mL (FW) at 30th day (Table 2), 13.4 times higher than that of the initial inoculums. The volume of culture medium decreased to almost one third of the original volume by the time of collection, likely due to the intake of water and nutrients by growing adventitious roots.

With the prolonging of culture time, the adventitious roots in liquid media became tangled and formed a round shape of roots. The top and bottom regions of the adventitious root proliferated significantly and appeared yellowish white in color and the inside of the adventitious root differentiated into old and senescent tissues in brownish yellow (Fig. 2), consistent with previous observations for other plant species (Min *et al*., 2007). This could be due to the exposure of periphery tissues to sufficient nutrients and oxygen; by contrast, the inside of cultures was of restricted supply of nutrients and oxygen.

Correlation of accumulation of total saponin with culture time: Overall, the pattern of saponins accumulation in adventitious roots displayed a "V" trend. An increase in the amount of saponins was initially observed for the first 7 days of incubation (13.4 mg/g dry mass at 7th day). Beyond this period, the content of saponins showed a gradual decrease and arrived at the minimal value at 24 days and then followed by a rise in the amount of saponins accumulation up to 33 days of incubation (Fig. 3). Taken together, the data of both Table 2 and Fig. 3 demonstrated that there is a reciprocal correlation between the secondary metabolite accumulation and the growth period of adventitious roots. And these results further suggested that the biosynthesis of saponin in the adventitious roots of *P. tunicoides* was in a mode of cell differentiation dependence.

Comparison of total saponins between liquid-cultured adventitious roots and other materials: The present study indicated that the adventitious roots of *P. tunicoides* resulted in a higher production of saponins in liquid culture (16.33±0.85 mg/g DW) compared to callus (11.71±0.90 mg/g DW) (Wei *et al*., 2010), with a comparable production of that for sterile seedlings (17.85±1.25 mg/g DW). Also, the adventitious roots got the highest relative growth rate among these different cultures or explants and achieved the best yield of total saponins per liter basis (Table 3). In addition, liquid culture of *P. tunicoides* brought about the excretion of some saponins into the media during liquid culture of adventitious roots of *P. tunicoides*. Taken together, these data suggest several advantages to use adventitious roots of *P. tunicoides* for the production of active compounds with medicinal value in the liquid system developed in this study.

Effects of a biotic elicitors on the accumulation of biomass and secondary metabolites: Although the possibility of enhancing accumulation of secondary products by the addition of biotic or abiotic elicitors to

Table 2: Biomass and growth of adventitious roots in liquid 1/2 B_5

Time of culture (days)	DW (g/L)	Growth ratio	Biomass productivity between two checked intervals (gDW/L/d)	Final biomass productivity (gDW/L/d)
0	1.120	-	-	-
7	1.650	0.467	0.075	-
15	5.680	2.442	0.504	-
22	12.07	1.125	0.913	-
30	13.45	0.114	0.173	0.411

This represents a typical result of three experiments

Table 3: Comparison of total saponins content between different cultures and explants

Sample type	Content of total saponins (mg/g DW)	Relative growth rate	Yield of total saponins (mg/L medium)
Sterile seedlings	17.85±1.25	0.028±0.0079	401.630
Callus	11.71±0.90	0.034±0.0065	263.750
Adventitious roots	16.33±0.85	0.182±0.0082	2041.25
Liquid media	0.540±0.10 (mg/mL)	-	540.000

This represents the mean value of three experiments plus S.D.

Table 4: Effects of elicitors on the growth and accumulation of adventitious roots of *P. tunicoides*

Time of addition of oxalate (day)	Biomass of adventitious roots (g/L DW)	Contents of total saponins (mg/L)
0	8.12±0.250	9.4±0.400
5	9.41±0.320	10.8±0.62
10	12.50±0.58	13.2±0.82
20	14.20±1.20	15.6±1.54

This represents the mean value of three experiments plus S.D.

the medium has been extensively studied in plant cell cultures, there are only a few reports of elicitors being applied to hairy root cultures. Oxalic acid was a kind of secondary metabolites in plant cells and has been proven to be an efficient abiotic elicitor for the induction of tanshinone and depsides in *Salvia miltiorrhiza* suspension cells (Zhang *et al*., 2009), a series of pathogen resistance-related enzymes (Zhang *et al*., 1998a, b). Here, we first described the profile of the growth of adventitious roots affected by oxalic acid application in the mode of liquid culture. As shown in Table 4, it indicated clearly that the oxalic acid-caused effect on the growth rate of adventitious roots depends on the stage of growth at which they were treated. It retarded the growth of adventitious roots when oxalic acid was fed to the culture medium at the initial stage of culture, however, it could successfully stimulate a remarkable accumulation of total saponins without adverse effects on the growth of adventitious roots after addition of oxalate at the late stage of culture period (e.g., at the 20^{th} day of culture). This result seems to be explained by the observations that the slight toxicity to the growth of plant cells caused by low concentration oxalate and its systematic induction on secondary metabolisms in whole seedlings (Zhang *et al*., 2009; Zhang *et al*., 1998a, b).

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