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Research Article

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Induction and characterization of callus from *Psammosilene tunicoides* hairy roots

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ABSTRACT

In our previous study, we reported the success of inducing hairy roots from the P. tunicoides leaves by infection of Agrobacterium rhizogenes ACCC10060. In this paper, we further reported the induction of callus from the induced hairy roots of P. tunicoides. Results showed that 6-BA and 2,4-D improved the dedifferentiation of hairy roots by inhibition of its growth, while NAA and KT both had no detectable effect on this aspect. Rational combination of 6-BA and 2,4-D therefore led to successful dedifferentiation of the induced hairy roots into callus. Agropine analyses demonstrated that hairy root-derived callus still remained the ability of agropine biosynthesis, which suggested that the callus didn't lose its transformed Ri genes after dedifferentiation. More important, the content of total saponins within hairy root-derived callus appeared indistinguishable from that of the parental hairy roots, and four times higher than that of normal callus (induced from normal explants, such as leaves, root tips, flowers etc.), indicating the strong ability of synthesizing secondary metabolites within hairy root-derived callus. Taken together, these results suggested that the large scale culture of hairy root-derived callus would be an efficient alternative to the field cultivation or wildlife resources of P. tunicoides for the production of useful natural compounds.

Keywords: Psammosilene tunicoides, hairy root callus, plant growth regulators optimization, orthogonal test

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INTRODUCTION

Psammosilene tunicoides W. C. Wu et C. Y. Wu is of the *Psammosilene* genus of *Caryophyllaceae*. It mainly grows in the South-Western regions of China, and is primarily distributed at an elevation of 1600-3200 m in the crevices of sunny rocks or weak acid sandy soil (1). It has been claimed that its tuberous root exhibits various therapeutic effects, including eliminating rheumatism, ease pain and expelling stasis (2). Although triterpenoid saponins, organic acids, cyclic peptide, lactam and some amino acids have been identified as the active components of *P. tunicoides* , the total saponins of this plant appear the main active ingredients with activity of pain-relieving and anti-inflammatory (3). In recent years, destruction of habitat, lasting and illegal collection have led to supply shortage of *P. tunicoides* in China and other Asian countries. Moreover, it usually takes 3-4 years for wild *P. tunicoides* plants from seed germination to final harvesting. Hence, it's urgent and necessary to seek alternative to the wild resources of *P. tunicoides* for production of natural compounds, such as commercial culture of plant cells, tissues and organs *in vitro*.

However, many reports have described that yields of desired products were very low or even undetectable in dedifferentiated cells such as callus tissues or suspension cultured cells (4). In order to obtain products in concentrations high enough for commercial manufacturing, it's necessary to take some measures to stimulate or restore biosynthetic activities of cultured cells (5). In this case, hairy roots, the result of genetic transformation by

Agrobacterium rhizogenes, have attractive properties for secondary metabolite production. *A. rhizogenes* is a gram-negative soil-born bacterium causing hairy root in many plants (6). The hairy roots induced by *A. rhizogenes* have a number of advantages, including fast growth in hormone free media, strong root branching, genetic and biosynthetic stability, plagiogeotropism, and biosynthetic capacity comparable to native plant roots (7). Now, it's becoming a promising technique in the field of medicinal plant cell engineering. The hairy roots of *P. tunicoides* has been reported to display identical saponines profile to wild type roots, and often accumulate phytochemicals at the same or higher levels than undifferentiated cells cultures, and non-transgenic root cultures (8). However, it is not known if these induced hairy roots could be further induced into callus, and little was known about the accumulation of secondary metabolites in the hairy root-derived callus. It is of significance to know whether the induced callus still retains the properties of hairy roots, such as stable biosynthesis of secondary metabolites, fast growth in phytohormones-free media. If so, this kind of special callus with *Ri* gene would be cultured in large scale for production of desired products, on the other hand, could be a potential alternative source of suspension culture cells. In this study, we focused on establishing an efficient protocol and procedure for callus induction and proliferation from hairy roots, and a comparison of biomass growth and saponines accumulation capability among hairy roots, hairy root-derived callus and normal callus. The results here would be helpful to evaluate the feasibility of secondary metabolites production of *P. tunicoides* by hairy root-derived callus on a large scale.

EXPERIMENTAL SECTION

P. tunicoides hairy roots were used as explants for callus induction. 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, the detailed were according to the procedure described by Li et al. (9).

Induction of hairy roots-derived callus

L_{16} (4^4), four factors and four levels, was introduced to examine the effects of various plant growth regulators (PGRs) on the induction and multiplication of callus from hairy roots by the orthogonal design. Four PGRs including 2,4-D （A）,NAA（B）,6-BA（C）and KT（D） were selected and added into the MS, and the factors and levels of experiments were listed in Table 1.

Tab. 1. Factors and levels of PGRs for induction and multiplication of hairy root-derived callus

levels	A	B	C	D
	2,4-D/（mg·L^{-1}）	NAA/（mg·L^{-1}）	6-BA/ （mg·L^{-1}）	KT/ （mg·L^{-1}）
1	0	0	0	0
2	0.5	0.5	0.5	0.1
3	1.0	1.0	1.0	0.2
4	2.0	2.0	2.0	0.5

Tips of fresh *P. tunicoides* hairy roots were cut into segments of 2~3 cm, and then placed on the MS supplemented with various concentrations of PGRs. All samples were divided into five groups, and each contained 75 segments of root tips. After culture of 28 d under 25±1℃and darkness, explants with formation of callus were counted and thus the induction rate of callus was calculated as formula (1).

The callus with properties of yellow appearance, fast-growing, loose and fragile texture was chosen to be sub-cultured on MS supplemented with various PGRs (as listed in table 1) ; samples were divided into five groups, and the inoculums of each flask was about 0.5-1.0 g (FW) . After 28d of culture under 25±1℃ and darkness, the relative growth of callus was calculated as formula (2).

Callus induction rate (%) =number of explants with callus /total explants *100% (1)

Relative growth rate=t^{-1}*ln (W_2/W_1) (2)

W_1: initial weight of callus (FW, *g*)
W_2: harvested weight of callus (FW, *g*)
t: culture time (d)

Identification of opines by TLC technique

Samples of hairy roots, hairy root-derived callus, original plant, and original plant-derived callus were dried to a constant weight at 60℃, and then ground into fine powder, respectively. Extraction and identification of opines was conducted according to Li *et al*.(9).

Extraction and determination of total saponins

Collections of different cultures were dried at 60°C to a constant weight 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 to dryness, a weighed pellet was dissolved in water.

Total saponins content was determined according to the procedure previously published with subtle modification (10,11). 5 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.

RESULTS

Effects of PGRs on the dedifferentiation of hairy roots

To determine whether there was a preference to cytokinin or auxin for callus induction from hairy roots of *P. tunicodes*, hairy roots were inoculated in MS media with different hormone combinations and concentrations. It's demonstrated that the hairy roots grew fast on PGRs-free culture media, and no visible callus was found on the surface of hairy roots (Fig.1-A). In the presence of 2,4-D or NAA but in absence of 6-BA (a kind of cytokinins), a number of callus could be induced successfully from the hairy roots (Figs.1.-F,K,P) . On other media supplemented with 6-BA, in contrast, few or no hairy roots exhibited visible callus (Figs.1-D,G,J,M).These results indicated that 6-BA exhibited inhibitory effects on the growth of hairy roots during dedifferentiation of hairy roots, and subsequentlypromoted the initiation and growth of callus from the hairy roots. It was worth to be noted that the callus induced by high level of 6-BA possessed traits of dense and hard texture (figs.1.D,G,J,M) .

On the media without 2,4-D, the rate of dedifferentiation of hairy roots was lower than other media containing 2,4-D (Figs.1.-B,C,D), which demonstrated that 2,4-D might play active roles in the dedifferentiation of hairy roots. Relationship between callus induction rate and 2,4-D concentration presented a "bell" curve, with the rate of induction ascended with the increase of 2,4-D concentration in the low range and then declined, suggesting the induction could be inhibited by high concentration of 2,4-D.

As to other PGRs, NAA had certain facilitation to the dedifferentiation of hairy roots, just weaker than that of 2,4-D in this respect. Although KT, a kind of cytokinins, had no obvious effect on the dedifferentiation of hairy roots, it could function during its subsequent growth callus.

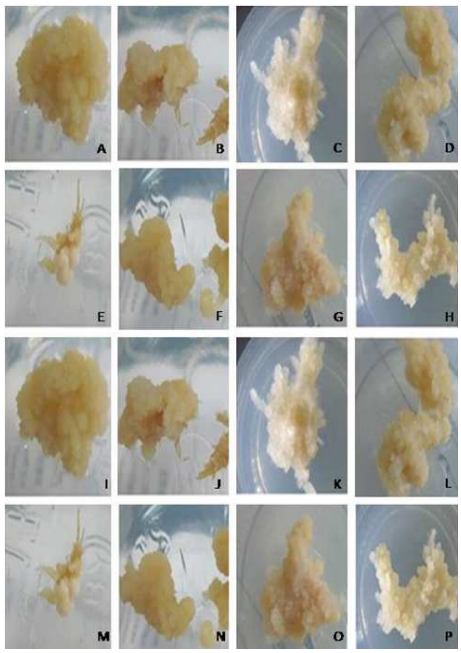

Fig.1 Typical photographs of hairy root-derived callus induced by PGRs

The combinations of PGRs in figs.1 were shown in Table 1

The results of orthogonal design of PGRs affecting the callus induction from hairy roots were shown in table 2. Based on the reference index of both induction rate and relative growth rate of callus, the extreme difference analysis displayed the difference in the effects on the induction and proliferation of *P. tunicoides* callus by different PGRs (fig.1). In terms of callus induction rate, four PGRs showed the impact on the dedifferentiation of hairy roots with sequences as 2,4-D, 6-BA,NAA and KT, and the best combination of PGRs was 2,4-D 1.0 mg/L + 6-BA 1.0 mg/L+ NAA 2.0 mg/L; On relative growth rate, the PGRs were listed as 2,4-D、6-BA、NAA、KT, and the formula of 2,4-D 0.5 mg/L+ 6-BA 1.0 mg/L + NAA0.5 mg/L + KT 0.2 mg/L was tested to be the best.

Tab. 2 The orthogonal design based on L_{16} (4^4) and the results of callus induction rate and relative growth rate

No.	2,4-D	NAA	6-BA	KT	Induction rate %	Relative growth rate
1	0	0	0	0	0	0.03346
2	**0**	0.5	0.5	0.1	25.6	0.03770
3	0	1.0	1.0	0.2	26.8	0.03806
4	0	2.0	2.0	0.5	15.2	0.01416
5	0.5	0	0.5	0.2	78.5	0.05761
6	0.5	0.5	0	0.5	46.5	0.05960
7	0.5	1.0	2.0	0	70.5	0.03578
8	0.5	2.0	1.0	0.1	87.6	0.06190
9	1.0	0	1.0	0.5	90.1	0.06276
10	1.0	0.5	2.0	0.2	62.3	0.03612
11	1.0	1.0	0	0.1	60.5	0.04841
12	1.0	2.0	0.5	0	90.1	0.02478
13	2.0	0	2.0	0.1	30.1	0.01311
14	2.0	0.5	1.0	0	68.3	0.03623
15	2.0	1.0	0.5	0.5	65.1	0.02355
16	2.0	1.0	0	0.2	30.6	0.03206
K_1	67.6	198.7	137.6	228.9		
K_2	283.1	202.7	259.3	203.8		
K_3	303.0	222.9	272.8	198.2		
K_4	194.1	223.5	178.1	216.9		
R	235.4	24.8	135.2	30.7		
K_1	0.123	0.167	0.174	0.130		
K_2	0.215	0.170	0.144	0.161		
K_3	0.172	0.146	0.199	0.164		
K_4	0.105	0.133	0.099	0.160		
R	0.110	0.037	0.100	0.034		

To further explore the effects of PGRs concentration on the above reference index, variance analysis was performed and the results were shown in table 3 and 4. It was found that 6-BA exerted significant effect on callus induction, and 2,4-D showed remarkable significance. However, both NAA and KT had not any significant effects on the above index. According to the above analysis data, the best combination was $A_3B_4C_3D_1$ for callus induction, and the best was $A_2B_2C_3D_3$ for relative growth rate.

Fig.2 TLC analysis of opine in hairy root callus. Original plant 2.Callus of original plant 3.Hairy roots 4.Hairy root-derived callus5. Bacteria liquid

Identification of opines in hairy root-derived callus

To determine whether the callus still remain the inserted sequence by *Ri* or not, the opines was examined by TLC technique. Results showed a positive reaction, by contrast, the samples of bacteria liquid, *P. tunicoides* seedlings and

normal callus had no detectable reaction spots (indication of opines)(fig.2). These results suggest that the transformed callus still carried the Ri sequence which has been inserted into the parental hairy roots.

Tab.3 Analysis of variance（inducing rate）

Source of variance	Sum of deviations square	Degree of freedom	*F* value	significance
A	8626.99	3	71.22	**
B	128.61	3	1.06	
C	3154.62	3	26.04	*
D	141.82	3	1.17	
e	121.13	3		

*$F_{0.05}$（3，3）=9.28 **$F_{0.01}$(3，3) = 29.5

Tab.4 Analysis of variance（relative growth rate）

Source of variance	Sum of deviations square（$\times 10^{-3}$）	Degree of freedom	*F* value	Significance
A	1.844	3	13.37	*
B	0.2312	3	1.68	
C	1.3789	3	9.99	*
D	0.1871	3	1.36	
e	0.1379	3		

*$F_{0.05}$（3，3）=9.28 **$F_{0.01}$(3，3) = 29.5

Evaluation of accumulation of total saponines in hairy root-derived callus

To evaluate the feasibility of saponines production by hairy root-derived callus culture on a large scale in the future, the comparison of total saponines content between hairy root-derived callus and other tissues was carried out (tab.5). Both the accumulation of saponines and the relative growth rate of hairy root-derived callus, two key reference indexes, were similar to that of hairy roots at the 28th day of culture, which indicates, at least in part, that the former still possesses the merits of fast growth and high accumulation of secondary metabolites of hairy roots. More importantly, compared with the normal callus, the hairy root -derived callus displayed a higher growth rate and contained a higher amount of saponins.

Tab.5 Total saponins content of *P. tunicoides* in different tissues or cultures

Tissue types	Relative growth rate	Saponin content (/%)
Callus	0.04	0.37
Hairy roots	0.07	0.84
Hairy root-derived callus	0.07	0.84

DISCUSSION

According to the theory of cell totipotency, any part of mother plant could be taken as explants for callus induction, such as root, leaf, stem, flower and fruit. However, hairy roots, as special explants, are characterized by high growth rate, genetic stability and growth in hormone free media, and more importantly, levels of secondary metabolites comparable to that of intact plants (12). Hence, it's reasonable to hypothesize that there exist certain differences in capacity of callus induction between hairy roots and its counterpart explants of mother plant. The analyses of orthogonal test indicate that four PGRs affect the dedifferentiation of *P. tunicoides* hairy roots with various abilities ranking as 2,4-D＞6-BA＞NAA＞KT, and variance analyses show that both 6-BA and 2,4-D have significant effects on the induction of callus. The combination of 6-BA and 2,4-D could induce callus from the hairy roots maximally confirmed by these results. The media supplemented with 2,4-D resulted in the formation of callus from hairy roots easily; however, the induction rate of callus might decline due to the continuous growth of hairy roots during the callus induction. By contrast, 6-BA could bring about high callus induction rate because of its inhibition on the growth of hairy roots. It's also observed that the hairy root-derived callus would re-differentiate into hairy roots autonomously on the phytohormone-free media during subculture of callus; accordingly, it's necessary to use certain phytohormones for sustainable growth of callus. From the orthogonal data, 2,4-D was the most efficient in terms of callus proliferation followed by 6-BA, NAA and KT in sequence, which was in consistent with the analysis of induction rate by PGRs tested. Exogenous application of cytokinin and auxin with a specific ratio may help to maintain the required ratio which favored the callus production, as documented for other plant species (13). The variance analyses further confirmed that both 2,4-D and 6-BA have significant effects on the hairy root-derived callus proliferation, displaying far fast growth over normal callus during subculture. The results here indicated that the requirement for PGRs were different for callus induction between hairy roots and normal explants, since a number of evidences have demonstrated that it could be induced easily from normal explants even on PGRs free MS.

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REFERENCES

[1]Wang,X., Qiu, D., and Jiang, C.: *Chinese Journal Basical Medicine Tradit. Chin. Med*. 8,pp.877-881,**2002.**
[2]Deng, X., Liu, X.-X., Zhu, D.,and Wang, Q.:, *Chin. J. Nat. Med*., 7, pp.101-104,**2009**.
[3]Wang, X., Zhang, Y., and Xu, J.: *Chin. J. Chin. Mater. Med*., 31, pp.419-421,**2006**.
[4]DiCosmo, F., and Misawa, M. : *Biotech*, 3(3),pp.425-53,**1995**.
[5] Kim, Y.-J., Wyslouzil, B.-E., and Weathers, P.-J.: *Biol. Plant*, 38,pp.1-10,**2002**
[6]Tsuro, M., Kubo, T., and Shizukawa, Y.: *Plant Cell Tiss. Org. Cult.*, 81,pp.175–181 ,**2005**.
[7]Ono, N.-N., and Tian, L.: *Plant Sci*., 180(3), pp.439-446,**2011**.
[8]Liu, T.-X., Geng, S.-H., Zhang, Z.-S.:. *Mater. Med. Res*., 22,pp. 2235-2236,**2011**.
[9]Li ,J.-B.， ,Liu, T.-X.， Wang, P.-Z.， and Zhang ,Z.-S.: *Chin. J. Chin. Mater. Med*., 36(5),pp.547-550,**2011**.
[10]Zhang, Z.-S., Yu, Z.-Y., Jin, Z.-X.: *Adv. J. Food Sci. Techn*., 5(2), pp.127-131,**2013**.
[11]Zhang, Z.-S., and Wang, Z.-Q.: *J. Dalian Polytech. Univ.*, 28,pp.174-177,2009.
[12]Srivastava, S., and Srivastava, A.-K.: *Cri. Rev. Biotech*., 27,pp.29-43,2007.
[13]Baque, M.-A., Hahn, E.-J., and Paek,K.-Y.: *Plant Biotech. Rep*., 4,pp.109–16,2010.