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Induction and characterization of adventitious roots directly from the explants of *Panax notoginseng*

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Abstract

Adventitious roots from leafstalks and lateral roots were obtained directly from explants of *Panax notoginseng*. The lateral root explants were more sensitive to the induction of adventitious roots using indole-3-butyric acid. HPLC analysis of saponins extracted from the adventitious roots indicated that several protopanaxatriol saponins were present but ginsenoside Rd was missing, compared with the saponins extracted from the raw herbs. The dry weight of primary adventitious root culture of *Panax notoginseng* increased 5.25 times during multiplication in a classical shaking-flask system, suggesting that it is a culture system with great potential for scale-up.

Introduction

Notoginseng (*Panax notoginseng* F.H. Chen), also known as sanqi, is a well-known Chinese medicinal plant belonging to the family Araliceae, and distributed mainly in the narrow belt of southwest China. It is effective in restoring homeostasis, blood-nourishing and treating coronary thrombosis (Xiao 2002, Jia & Gao 2003). The ginsenosides and notoginseng saponins are important active components of the roots of *P. notoginseng*, which have a wide range of therapeutic effects such as immunoregulatory, anti-fatigue, hepato-protective, and anti-cancer functions (Han & Zhong 2002).

Callus induction and suspension cell culture systems have been studied (Zheng & Liang 1978, Zheng *et al.* 1989, Zhong & Zhu 1995, Zhang & Zhong 2004) with the aim of increasing the secondary metabolism. However, the large-scale suspension cell culture has been difficult due to poor productivity and instability of de-differentiated cells when in culture (Payane *et al.* 1991). The potential of root cultures from various species of medicinal plants has been explored (Yu *et al.* 2002). One of the techniques is the hairy root culture but this can be sub-optimal for commercial production because the extracts contained opine-like compounds, which are potentially harmful for mammalian cells (Yoshikawa & Furuya 1987). Recently several research groups have studied adventitious root culture (Hahn *et al.* 2003, Seo *et al.* 2003). In the present study, pioneer experiments on adventitious root culture of *P. notoginseng* were conducted, where some important factors affecting the induction of adventitious root were evaluated.

Materials and methods

Plant material

Three-year-old fresh notoginseng plants (*P. notoginseng* F.H. Chen), provided by Notoginseng Scientific Technology Research Institute (Wenshan county, Yunnan province, China), were used.

Preparation of explants

The whole plant of three-year-old notoginseng was thoroughly rinsed under running tap water before removing the dust from the root with a brush. The plant was then dipped in a detergent for 2–3 h, flushed with water, and the external water removed with filter paper. The surface of explant leaves, leafstalks, stems, roots and lateral roots were sterilized with 75% (v/v) ethanol for 30 s, immersed in 1% (w/v) sodium hypochloride containing two drops of Tween 20 for 5–20 min, and then rinsed with distilled water five times. The sterilized explants were cut to approx. 1 cm length for lateral roots, 0.5–0.8 cm for stems and leafstalks, 1 cm diam. for leaves and 0.1 cm^3 for roots.

Induction of adventitious roots from explants on solid medium

The parts of explants processed as above were placed in solid Murashige and Skoog medium (MS) containing 0.45% (w/v) agar to induce adventitious roots. The solid MS basal medium was supplemented with different concentration of phytohormone. The pH of the medium was adjusted to 5.8 before autoclaving (121 °C for 15 min). The cultures were incubated in the dark at 25 ± 1 °C.

Identification of micrography on adventitious root

The adventitious roots initiated from lateral roots and leafstalks were used as materials for paraffin section. Tissue sections 20 μm obtained by the general paraffin section technique were inspected under a microscope.

Culture of adventitious roots and sample preparation

A liquid culture system was established by inoculating 0.5 g (fresh wt) root into 250 ml Erlenmeyer flasks containing 100 ml liquid 1/2-strength MS medium supplemented with 3 mg indole-3-butyric acid (IBA)/l and 30 g sucrose/l. The flasks were shaken at 100 rpm at 25 ± 1 °C in the dark. The freshly harvested roots were weighed weekly.

The harvested roots were dried *in vacuo* (−0.1 MPa) at 50 °C to constant weight and extracted with methanol (25 ml methanol per 1 g dry powder) in an ultrasonic bath at maximum sonification (250 W) for 30 min. After filtration, the extract was evaporated to dryness *in vacuo*, then dissolved in 10 ml HPLC-grade water. The water-soluble extract was passed slowly through a HZ-801 adsorbent resin, eluted with 30 ml 50% (v/v) aqueous ethanol. The elution was concentrated *in vacuo* and transferred to a 10 ml volumetric flask. The saponin fraction was used for total saponin content determination and HPLC analysis.

Saponin analysis

The saponin fraction obtained was allowed to react with 5% (w/v) vanillin in acetic acid and perchloric acid at 60 °C, forming a purple product with maximum absorbancy at 560 nm.

An analytical procedure, with an Agilent 1100 liquid chromatograph (Palo Alto, CA, USA) equipped with quaternary gradient pump and a reversed-phase column (Elite, Hypersil ODS, 250×4.6 mm, I.D., 5 μm), was employed for qualitative and quantitative determination of saponin. The binary gradient elution system, at 1 ml min^{-1}, consisted of water (A) and acetonitrile (B), and separation was achieved using the following gradient: 0–15 min, 20% B; 15–40 min, 20–33% B; 40–60 min, 33–50% B; 60–70 min, 50–75% B; 70–75 min, 75–100% B. The column was kept constant at 35 °C. The eluate was monitored at 203 nm and the ginsenosides (Rg1, Re, Rb1, Rd) and notoginsenoside R1 were identified using standards (from the National Institute for the Pharmaceutical and Biological Products, Beijing, China).

Results and discussion

Effect of different explants on adventitious root organogenesis

Different parts of the explants demonstrated significant differences in the formation of

adventitious roots. The lateral root explants yielded calli which appeared yellow and grainy after 10 days' induction; after 35 days white or yellowish adventitious roots appeared. At 14 days, the surface of leafstalk explants produced compact white, semi-transparent calli, which then formed many spherical white callus lumps, and extruded white semi-transparent adventitious roots from the callus lumps at 45 days. These adventitious roots were gradually covered with large numbers of characteristic short white root hair. The frequency of adventitious roots induced from lateral root explants (44%) is appreciably higher than that from leafstalk explants (37%). The explants of leaves, stems and roots failed to initiate root organogenesis.

Induction of adventitious root is the key step the root culture *in vitro*. Lateral roots explants appeared to be the more productive in plant parts for initiating adventitious root organogenesis than leafstalks and others, presumably due to the lateral root being a tender organ possessing the property of rapid growth and incomplete differentiation.

Effect of exogenous phytohormones on adventitious root initiation

Table 1 shows that IBA was the only phytohormone initiated adventitious root organogenesis from explants when 2,4-dichlorophenoxyacetic acid (2,4-D), IBA or kinetin was used individually. The effects of IBA alone and IBA combined with kinetin had no significant difference on callus formation and adventitious roots formation. The root organogenesis appeared to be non-productive in the 2,4-D and kinetin groups. The addition of 2,4-D restrained the morphogenesis of adventitious roots from explants with or without IBA.

Table 1. Effect of exogenous phytohormones on adventitious root organogenesis.

Phytohormones (mg l^{-1}) [a]	Frequency for induction (%) [b]	
	Adventitious root	Callus
2,4-D (1)	0 [c]	13 ± 2
IBA (3)	49 ± 3	83 ± 4
Kinetin (0.1)	0	0
IBA (3) + Kinetin (0.1)	46 ± 3	79 ± 5
2,4-D (1) + IBA (3) + kinetin (0.1)	0	65 ± 3

[a]2,4-D and IBA represent 2,4-dichlorophenoxyacetic acid and indole-3-butyric acid respectively. The number inside the bracket behind phytohormone represents the concentration of the corresponding phytohormone.
[b]Data represent the mean value ± SE of three independent experiments.
[c]"0" represents that adventitious roots or calli are failed to be induced from explants.

Somatic plant cells may form adventitious embryos, roots and shoots with the proper stimuli, the process of which consists of three phases: dedifferentiation, induction and realization (Klerk *et al.* 1997). During the induction phase, each pathway requires specific hormonal triggers. IBA is an effective hormonal trigger which stimulated the formation of *P. notoginseng* adventitious roots, whereas 2,4-D initiated callus formation but not adventitious roots. IBA was thus favorable for induction of the adventitious root in *P. notoginseng* culture. This is partially supported by similar studies on *Panax ginseng*, where IBA was also effective (Son *et al.* 1999, Hahn *et al.* 2003, Seo *et al.* 2003).

Identification of adventitious root with micrography

Sections of the adventitious roots were stained with Safranine T and Fast Green to distinguish different tissues. Adventitious roots possessed vessel segments for transporting water and inorganic elements. These vessel segments are formed in root of mature plants therefore the micrography of adventitious roots showed that the characteristic morphology of mature roots had been established.

Primary culture of adventitious root and total saponins production

The growth and secondary metabolite production patterns of adventitious root cultures are shown in Figure 1. Saponins increased up to the end of growth (35 days) reaching 46 mg g^{-1} dry wt. The dry weight of the adventitious roots increased 5.25 times (Figure 1), suggesting that this is a promising culture system for large-scale commercial production.

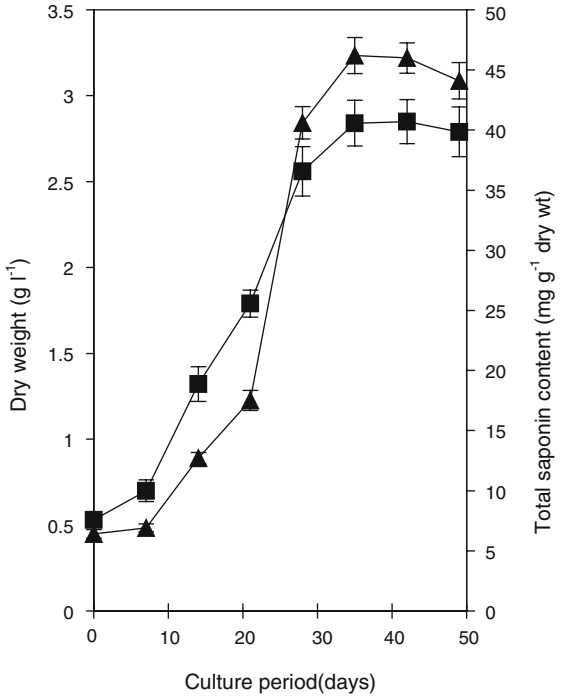

Fig. 1. Time course of adventitious root growth and total saponin production of *Panax notoginseng* in 250 ml Erlenmeyer flasks with 100 ml 1/2-strength Murashige and Skoog liquid medium supplemented with 3 mg indole-3-butyric acid/l. ■ = dry weight of adventitious roots harvested, ▲ = saponin production of dried adventitious roots. All experiments were repeated three times. Data were expressed as mean ± standard error (SE).

Saponins HPLC profiles of adventitious roots

The HPLC profile of saponins extracted from the adventitious roots was somewhat different from that of raw herb extract of *P. notoginseng*. The main ingredients were ginsenosides Rg1, Rb1, Re, Rd and notoginsenoside R1, which is the characteristic saponin of *P. notoginseng* compared with *P. ginseng* and *Panax quinquefolium*. However, adventitious roots cultured *in vitro* had a significantly different profile and no ginsenoside Rd (Table 2) was detected. There was a decreased content of Rb1, and a number of new peaks in the HPLC eluate between 17 and 42 min, which indicated that many minor protopanaxatriol saponins were accumulating during the root culture.

Notoginseng contains more than 20 different ginsenosides, and the saponin contents are usually evaluated in two ways: the total saponin content and the contents of key ginsenosides. The total saponin content is more convenient for the comparison of the amount of saponin in different samples, whereas the distinction of total saponin contents and main saponin composition between *in vivo* and *in vitro* cultures reflects the change of metabolic network. Similar to the disappearance of Rd, Wang *et al.* (2005) also reported that notoginsenoside R1, a characteristic saponin of *P. notoginseng*, was not detected in their cell cultures.

A number of protopanaxatriol saponins were accumulated in addition to the main protopanaxatriol group saponins (such as ginsenosides Rg1, Re and notoginsenoside R1), suggesting that the metabolic flux of protopanaxatriol saponins may have increased. On the other hand, the metabolic flux of protopanaxadiol saponins (ginsenosides Rb1 and Rd) decreased or was inhibited. We anticipate that the contents of total saponins and main ginsenosides and notoginsenosides may be enhanced when the metabolic flux leading to protopanaxadiol and protopanaxatriol precursors is increased by optimizing culture conditions and using the signal molecules such as jasmonates or salicylate.

In conclusion, due to the drawbacks with large-scale culture of suspension cells and transformed root (hairy root) cultures, we believe that the adventitious root cultures may be a more favorable culture system for the production of

Table 2. Comparison of ginsenoside contents between adventitious roots cultured *in vitro* and native plant determined by HPLC.

	Protopanaxatriol (mg g^{-1} dry wt)			Protopanaxadiol (mg g^{-1} dry wt)	
	R1	Rg1	Re	Rb1	Rd
Native plant	6.4	18.8	4.3	16.4	1.8
Adventitious roots	3.1	6.1	1.2	2.1	– [a]

[a]No distinct characteristic peak was found.

notoginseng because of its simplicity, induction speed, and potential for scale up, based on our study of the adventitious root morphogenesis and culture of *P. notoginseng*.

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