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ORIGINAL RESEARCH PAPER

Growth kinetics and ginsenosides production in transformed hairy roots of American ginseng—*Panax quinquefolium L*

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Abstract A thin, profusely branched, fast growing hairy root line of *Panax quinquefolium* (American ginseng) was established by co-culturing epicotyl explants with a wild type strain of *Agrobacterium rhizogenes*. The transformed roots grew by over 10-fold from the initial inoculum within 8 weeks. The crude ginsenosides content in the roots was about 0.2 g/g dry wt level up to the 10th week of culture. Ginsenosides Rb2, Rd, Re, Rf and Rg1 constituted 47–49% of the crude saponin fraction between 6 and 8 weeks of growth whereas, Rc ginsenoside was accumulated only after 9th weeks when the biomass started receding. PCR amplification analysis of the hairy roots confirmed their transgenic nature by showing the presence of Ri-TL DNA with *rol*A, *rol*B and *rol*C genes in their genome.

Keywords *Agrobacterium rhizogenes* · Ginsenosides production · Growth kinetics · Hairy roots · Panax quinquefolium

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Introduction

The roots of *Panax* species (Family: Araliaceae) are valued for their triterpene saponins that have strong immuno-modulatory, adaptogenic and anti-ageing properties (Nocerino et al. 2000). *Panax ginseng* (Korean ginseng), *P. quinquefolium* (American ginseng), *P. notoginseng* (Chinese ginseng) and *P. japonicas* (Japanese ginseng) are the four major *Panax* species in the trade. Nearly 30 types of triterpene saponins, that are collectively referred to as ginsenosides, have been identified in ginseng roots. Among these, the ginsenosides Rb1, Rb2, Rc, Rd, Re, Rf, Rg1 and Rg2 are considered to be the most potent bioactives (Liang and Zhao 2008). Agri-based production of ginseng roots is difficult and time consuming due to extended seed dormancy, extremely slow plant growth and seasonal influences (Wu and Zhong 1999). Economic yield of roots with required ginsenosides concentration can be obtained only from a 4 to 7 year old crop (Johanssen 2006). To meet the increasing market demand of ginseng roots and pure ginsenosides, intensive efforts to produce them in vitro in callus, cell suspensions and root cultures are being pursued in many laboratories (Jeong et al. 2008; Kim et al. 2009; Mathur et al. 2000, 2003; Wu and Zhong 1999). *Agrobacterium rhizogenes*-mediated hairy root cultures, in particular, are of great relevance here because of their genetic stability and flexibility for bioreactor up scaling (Kim et al. 2009).

We report here for the first time the establishment, growth kinetics and ginsenoside productivity of an *A. rhizogenes*-mediated hairy root line of *Panax quinquefoilum*.

Materials and methods

Plant material and bacterial strains

Stratified seeds of *Panax quinquefolium* were purchased from Mr Scott Persons, Tuckasegee Valley (USA). They were soaked overnight in water, decoated and surface-sterilized with 1% (v/v) cetrimide for 5 min, ethanol for 1 min and then with 0.1% (w/v) $HgCl_2$ for 3–4 min. The surface-sterilized seeds were kept in a filter-sterilized antibiotic solution containing 500 μg chloramphenicol ml^{-1}, 500 μg neomycin sulfate ml^{-1} and 1,000 μg tetracycline ml^{-1} for 1 h before plating for germination on an agar-gelled MS (Murashige and Skoog 1962) basal medium. Suspensions of four *Agrobacterium rhizogenes* strains, A4, LBA9402, ATCC 11325 and ATCC 15834, were raised in YMB medium (Hooykaas et al. 1977) containing 50 mg kanamycin l^{-1}. Root, cotyledon, leaf and epicotyl explants (40–50 each) were excised from the 25 to 30 day old seedlings, pricked with needles dipped in 36 h old bacterial suspensions (OD 1.0 at 600 nm) and co-cultivated on MS medium at 28°C for 48 h. After co-cultivation the explants were repeatedly shifted every 4th day to fresh MS medium supplemented with 1,000 μg each of cephalexin and ampicillin ml^{-1} to kill LBA 9402 and A_4 strains or with 300 μg carbenicillin ml^{-1} to eliminate ATCC-15834 and ATCC-11325 strains of the bacteria. They were incubated in dark on an antibiotic-free MS medium for the emergence of roots. Untreated explants were used as control.

PCR analysis

Genomic DNA from transformed and non-transformed tissues was isolated following the method of Khanuja et al. (1999). Plasmid DNA of *A. rhizogenes* was isolated using a mini-prep plasmid kit and was used a positive control. Oligonucleotide primers and procedure described by Krolicka et al. (2001) were used for PCR detection of bacterial Ri plasmid genes *rol*A, *rol*B and *rol*C in the plant genome. PCR amplification with primers homologous to the sequence 5′-ATGTCGCA AAGGCAGTAAGC-3′ and 5′-AGTCGTACCTCGT TGGCAGC-3′ (GenBank accession number X12867) of v*ir*D gene present on Ri plasmid beyond the transferred T-DNA segment was also done to ensure that transformed tissue was free of *A. rhizogenes* cells; Amplification conditions used were: denaturation at 94°C for 5 min, 45 cycles of 1 min denaturation at 94°C, annealing at 45°C for 1 min and extension at 72°C for 2 min followed by the final elongation for 5 min. For amplification of *vir*D the annealing was carried at 55°C. The amplified products were separated on 1.2% (w/v) agarose gel in TEA buffer and stained with 0.5 mg ethidium bromide l^{-1} for viewing under UV light.

Growth and ginsenosides analysis

Approx. 0.2 g actively growing hairy roots (=15–20 mg dry wt) were inoculated in 40 ml hormone-free liquid medium consisting of one-fourth strength of inorganic salts and double strength of vitamin supplements of B5 (Gamborg et al. 1968) medium, 2 mg glycine l^{-1}, and 100 mg myoinositol l^{-1}. The cultures were shaken at 100 rpm for 12 weeks under diffused illumination (30 μmol m^{-2} s^{-1}). The fresh and dry biomass increase was measured weekly and growth was expressed as percent increase over the initial inoculum weight. The freshly harvested roots were processed for ginsenosides extraction and HPLC analysis as previously described (Mathur et al. 1994, 2003).

Statistical analysis

Mean data obtained on nine studied parameters namely biomass increase, crude ginsenosides content and percent concentration of Rb1, Rb2, Rc, Rd, Re, Rf and Rg1 ginsenosides in the crude ginsenoside pool) was subjected to ANOVA analysis (Lal et al. 1997). A minimum of three replicated cultures per treatment were raised and the experiments were repeated twice.

Results and discussion

The seedling-derived root, epicotyl, leaf and cotyledon explants of *P. quinquefolium* showed differential susceptibility for transformation when co-cultivated

with four *A. rhizogenes* strains. Highest transformation frequency (46.8%) was observed when epicotyls was infected with LBA9402 strain, followed by leaf and cotyledon explants with LBA9402 > ATCC158347 > A4 > ATCC11325. Root explants did not respond to any bacterial strains. Co-cultivation with bacterial strains ATCC15834 and ATCC11325 induced only limited callusing at the pricked sites from the epicotyl tissue that did not grow further. Such differential explants versus strain susceptibility has also been demonstrated earlier in *P. ginseng* (Chen and Punja 2002). The unorganized callus mass originated from epicotyls infected with LBA9402 upon transfer to MS medium showed emergence of plenty of hairy roots. For further growth and proliferation the induced hairy roots were cultured in full, half and one-fourth strength of B5 and MS liquid media. The best proliferation was observed in one-fourth strength of B5 medium supplemented with double strength of vitamins, 2 mg glycine l^{-1}, and 100 mg myoinositol l^{-1} as was also shown for hairy roots of *P. hybrid* (Washida et al. 1998). The transformed hairy roots showed three distinct phenotypes in culture (Fig. 1a–c): thin and highly branched roots (TN line), thick and sparsely branched roots (TH-line) and, thick roots with excessive callusing at the distal ends (TH-C line). Such morphological differences were indicative of the occurrence of independent transformation events due to different insertion sites of plasmid TL-DNA in the plant genome, its copy number or T-DNA structure *per se* as proposed earlier (Tepfer 1990; Mallol et al. 2001). Out of the three different root lines, the TN line showed best proliferation and growth due to their healthier and better organized root tips and hence, was advanced to detail growth and metabolite profiling studies.

The transgenic nature of the regenerated roots was confirmed by PCR analysis. The primers, complementary to *rol*A, *rol*B and *rol*C genes of TL-DNA of the Ri plasmid generated amplification products of approximately 770, 530 and 300 bp with the genomic DNA of the TN hairy root line (Fig. 2a). No such product was obtained from the non-transformed tissue. The absence of amplification products of *vir*D primer (450 bp) in the transformed roots (Fig. 2b) further confirmed that the roots were bacteria-free and the target for the PCR amplification of *rol*A, *rol*B and *rol*C genes was T-DNA incorporated into the plant genome.

Growth kinetics and ginsenosides production in the TN line of hairy roots was analysed over a 12 week culture passage (Tables 1, 2). The root biomass accumulation started from 2nd week onwards, continued up to 8 week and then steadily declined. The cultures grew by over 4, 8 and 10 fold from the initial inoculum after 3rd, 5th and 8th week of culture cycle. The crude ginsenoside concentration, however, did not vary with culture age and was steadily maintained at around 0.2 g/g dry wt level during the growth cycle. The initial higher content of crude saponins (0.3 g/g dry wt) observed up to the first 2 weeks of culture cycle might be a consequence of carry over influence of the initial inoculum or lesser biomass gain during

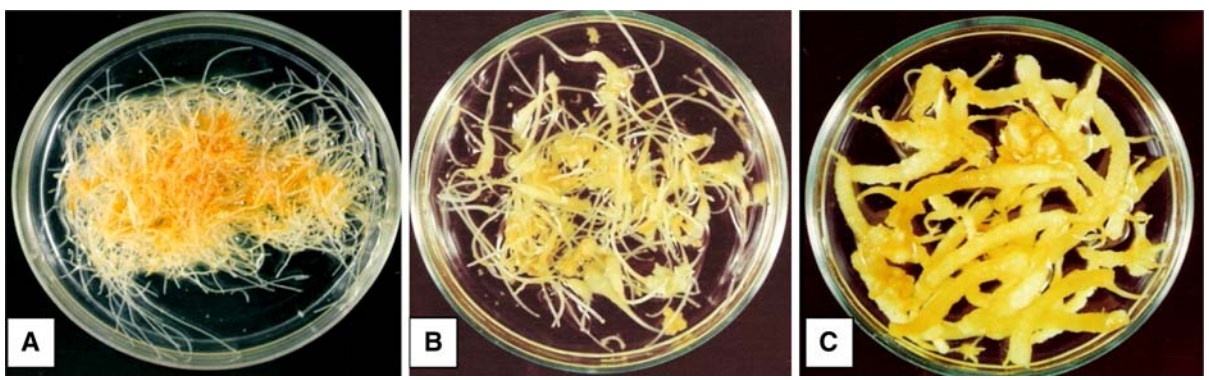

Fig. 1 Hairy roots of *P. quinquefolium* obtained from epicotyls explants transformed with *A. rhizogenes* strain LBA9402 exhibiting three different phenotypes during growth in B_5 medium. **a** Thin branched (TN line); **b** Thick and sparsely branched (TH-line); and **c** Thick roots with extensive callusing (TH-C line). All culture plates shown in the figure had a diameter of 90 mm

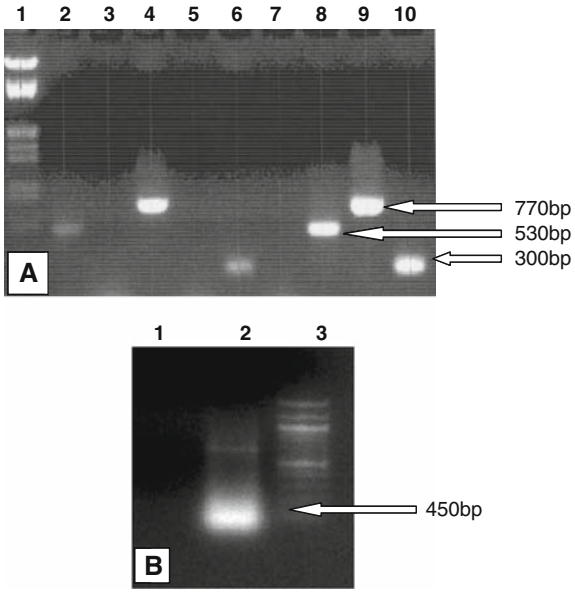

Fig. 2 PCR analysis of thin branched (TN) hairy root line of *P. quinquefolium*. **a** Amplified products corresponding to *rol*A (*lane 2*), *rol*B (*lane 4*) and *rol*C (*lane 6*) genes in the transformed roots; *lanes 3*, *5*, *7* showing the absence of plasmid genes in the non-transformed tissues (negative control); *lanes 8*, *9*, *10* showing amplification of *rol*A, *rol*B and *rol*C in the plasmid DNA (positive control); **b** Amplification of *virD* gene showing its absence (*lane 1*) and presence (*lane 2*) in the transformed tissue and plasmid DNA, respectively

this lag phase of growth as was suggested for hairy root cultures of *P. ginseng* (Yoshikawa and Furuya 1987; Mallol et al. 2001). HPLC analysis of the crude saponin extract of the hairy roots revealed that the three panaxatriol type ($Re > Rf > Rg_1$) and one panaxadiol (Rd) type of ginsenoside constituted 47–49% of the crude ginsenosides fraction between 6th and 8th week of growth. Rc ginsenoside was absent during active growth phase and appeared only after 9th week when growth declined. The Rb1 and Rb2 ginsenosides maintained a steady level of 2–3% of the total ginsenoside pool except a marginal increase between 7th and 9th week of culture cycle. The TN hairy roots line of *P. quinquefolium* developed in this study also maintained the ratio between Rb and Rg ginsenosides at > 1 as desired by the food and pharma industry for field grown ginseng roots. Interestingly, the total ginsenosides productivity of this line was 2.3 times more than a high yielding callus line of this species selected earlier in our laboratory (Mathur et al. 2003; US Patent No. 6326202).

To the best of our knowledge this is the first report of hairy root induction in American ginseng. The transformed roots established herein would be highly suitable for providing a sustainable production system

Table 1 Growth kinetics and ginsenosides content of thin branched (TN) hairy root line of *Panax quinquefolium* as a function of culture age

Culture age (week)	Dry wt increase over the initial inoculum (%)[a]	Total ginsenosides (g/g dry wt)	Ginsenoside fraction (% of the crude ginsenoside pool)						
			Rb1	Rb2	Rc	Rd	Re	Rf	Rg1
0	0	0.3	0.2	0.4	ab	tr	4.3	0.2	tr
1	45	0.3	0.3	0.6	ab	Ab	2.9	1.3	tr
2	152	0.2	1.1	1.5	ab	0.3	7.2	1.3	0.7
3	441	0.2	1.7	0.5	ab	0.3	8.5	2.1	0.7
4	827	0.1	2.7	2.7	ab	3.4	19.5	9.1	3.7
5	910	0.2	2.4	2.9	ab	3.9	20.0	9.8	4.6
6	933	0.2	2.6	2.9	ab	3.9	20.1	9.8	5.5
7	995	0.2	2.4	3.3	ab	4.7	23.1	10.7	5.2
8	1,078	0.2	2.8	3.7	ab	4.7	24.3	9.9	5.4
9	1,042	0.2	2.6	3.5	ab	4.7	24.9	9.3	4.7
10	830	0.2	2.6	1.8	3.4	4.9	28.7	9.4	4.6
11	754	0.1	2.6	1.6	1.6	0.2	9.5	0.6	3.4
12	534	0.1	2.2	0.6	0.8	tr	9.1	tr	3.1
CD at 5%	12.5	0.1	0.2	0.1	0.01	0.1	0.6	0.3	0.1
CD at 1%	16.9	0.2	0.3	0.1	0.01	0.2	0.8	0.4	0.1

Initial inoculum used in all the treatments was 0.2 g fresh wt (=15–20 mg dry wt)/culture in 40 ml medium

[a] Data are mean values of three replications

ab Absent, *tr* traces

Table 2 Analysis of variance (ANOVA) for the nine parameters studied in hairy root line of *Panax quinquefolium*

Source of variation	df	Mean sum of sqaure								
		Growth index	Crude ginsenosides content	Rb1	Rb2	Rc	Rd	Rf	Re	Rg1
Replicates	2	162	0.003	0.02	0	0	0.01	0.04	0.06	0.01
Treatments	12	465,609**	0.28**	2.4**	4.5**	2.9*	13.9*	63.6**	233.7**	13.4**
Error	24	55	0.01	0.01	0.001	0	0.01	0.03	0.12	0.002

The mean values are compared with mean sum of squares between replications and treatments

* $P < 0.05$; ** $P < 0.01$

df Degree of freedom

for ginseng root biomass and pure ginsenosides. Efforts towards its bioreactor scaling up are currently under way.

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