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In vitro root cultures of *Panax ginseng* and *P. quinquefolium*

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Abstract

The paper describes a procedure for the initiation, subculture and continued proliferation of adventitious roots of *Panax ginseng* and *Panax quinquefolium*, which resemble hairy roots. The technique took advantage of the high powerful activity of a new synthetic auxin: benzo[b]selenienyl acetic acid (BSAA). Such initiation from root explants was dependent upon the season, the type and concentration of auxin. The hairy-like roots of ginseng could be subcultured by transfer every 4 weeks to fresh liquid medium either in agitated Erlenmeyer flasks or in bioreactors. Optimal conditions for a continued multiplication (up to 14 per month) were determined. The only practical problem was the limitation of the fresh mass as inoculum: the multiplication rate decreased with the increased quantity of roots. It is postulated that a root growth inhibiting substance was released into the media by the proliferating ginseng hairy roots.

Abbreviations: BSAA – benzo[b]selenienyl acetic acid; IAA – indoleacetic acid; IBA – indolebutyric acid; MS – Murashige and Skoog medium (1962)

1. Introduction

Panax root has been used in Oriental medicine since ancient times. The crude root extracts are known to have tonic, stimulatory and adaptogenic properties [8] owing to the presence of a wide range of saponins and sapogenins [21]. In recent years, ginseng has also become a popular tonic and health food in Western countries. Therefore the demand for the plant has increased dramatically worldwide.

Ginseng is very expensive because of its long-term conventional (5–7 years) and troublesome cultivation. Therefore biotechnological alternatives, i.e., ginseng tissue cultures have been tested for *in vitro* ginsenoside production. Furuya and Ushiyama [3], Lee et al. [19] and Mathur et al. [22] were able to show that ginsenosides are produced in callus as well as in cell suspension cultures of *Panax ginseng* and *Panax quinquefolium*. The production of ginsenosides by transformed (with *Agrobacterium rhizogenes*) root cultures (hairy root cultures) was attempted [9, 10, 11, 14, 16, 17, 26, 27]. The advantage of hairy root cultures is that they retain differentiation while exhibiting growth rates comparable to those of plant cell suspensions. Unlike plant suspensions which often produce very small amounts of secondary metabolites, hairy root cultures can display high biosynthetic capabilities that are often comparable to those of normal roots [1, 2, 25]. The hairy roots of *Panax ginseng* synthesize the same saponins (ginsenosides) as those of the native root and up to about 2 times more than ordinary cultured roots [26]. Moreover, culture conditions [15] or elicitors [17] were able to enhance the contents of ginseng saponins produced by hairy root cultures. However, in practice genetically modified organisms or organs are not well accepted as food drugs. Therefore, the present aim was to establish hairy-like root cultures without transformation with *Agrobacterium rhizogenes*, which had never been attempted. The novel technique takes advantage of the

new synthetic auxin, benzo[b]selenienyl acetic acid, which has shown powerful activity in several auxin-controlled growth and development processes [4, 7, 12, 18].

2. Materials and methods

2.1 *Plant material*

One-year-old field-grown plants of *Panax ginseng* and *Panax quinquefolium* from the Belgian Ardennes were uprooted in September and stored in the glasshouse to December in sand. The whole roots were 2–3 cm long when 3 mm cross slices (diameter: 5 mm) were explanted.

2.2 *Sterilization and culture conditions*

Whole roots were surface sterilized by immersion successively in 70% ethanol for 5 min., in 1% teepol (S.A. Belgium Shell) and 3% sodium hypochlorite for 20 min. and finally for 30 sec. in 0.1% mercuric chloride and then rinsed three times 10 min. in sterile water. The MS [23] liquid medium was used as the basal medium. It was supplemented by an auxin [indoleacetic acid, IAA (from Merck, Darmstadt, Germany) or indolebutyric acid, IBA (from Duchefa, Haarlem, The Netherlands) or 3-benzo[b]selenienyl) acetic acid, BSAA (from Acros Organics, Geel, Belgium)], added before autoclaving (20 min., 121 °C, 1.2 Kg/cm^2). The media contained 30g/l sucrose (except when specified) and the pH was adjusted to 5.8. The cultures (liquid medium in Erlenmeyer flasks) were maintained in a growth chamber in the dark at 24 °C and agitated at 80 RPM. In some specified cases, the roots were enclosed in a gauze bag to permit an easy change of medium.

2.3 *Culture in bioreactor*

Inoculum was placed aseptically into a 2 l Biolafitte bioreactor containing 1.5 l of MS medium. The double walled glass cylinder ensumed that the temperature was maintained at 25 °C. The cultures were grown in the dark with an agitation of 80 RPM. PO_2 and pH was monitored during culturing.

3. Results

3.1 *Establishment of root culture*

Slices of one year-old roots were cultured in darkness in MS liquid medium supplemented with auxin (IAA, BSAA) at various concentrations (10^{-6} to 10^{-9} M). The same experiment was done in September and in December. Only in this last case and in the presence of BSAA at 10^{-6} M, 42% of the explants of *P. quinquefolium* and 34% of *P. ginseng* developed respectively 3 to 7 and 2 to 4 roots ($\pm$1 cm long) in one month. In the other conditions, no roots were formed but the explants swelled and some calli appeared at auxins concentrations 10^{-7} and 10^{-8} M.

The roots formed were cut from the explants and cultured in the same liquid medium MS supplemented with BSAA at 10^{-6} M. In one month, each root developed $\pm$10 new lateral roots. These lateral roots were 1 to 2 cm long.

For subsequent subcultures, three types of root cutting methods were tested (Table 1):

- Cutting up of each root separately (depilation) from the original root, with pincers.
- $\pm$10 Cuttings per original explant (rapid cutting), with razor blade.
- Transfer of the whole rooted explants to a new medium without cutting.

The simple transfer of the explants to a new medium did not induce new lateral roots. There was no increase of weight of the explant one month after transferring; browning of the explants occurred. The “depilation” was a labour consuming technique that did not give better results (multiplication rate and aspect) than the rapid cutting. The multiplication rate was up to about 15 but more for *P. ginseng* than for *P. quinquefolium*. The expression of the results by number or by FW was similar. In the next experiments, the explants were prepared by rapid cutting and the results of multiplication rate expressed by FW.

3.2 *Effect of exogenous auxins*

The effects of three exogenous auxins (IAA, IBA and BSAA) at various concentrations were tested on the multiplication rate of the hairy-like roots. Table 2 shows that the best results were obtained with BSAA at 10^{-6} M. Higher or lower BSAA concentrations decreased the multiplication rate. In the presence of BSAA at 10^{-5} M the roots were bigger, and conversely with BSAA at 10^{-7} M, they were

Table 1. Effect of the type of cuttings on multiplication rate (expressed by fresh weight and by number of new roots) of roots (0.4 g l^{-1}) of *Panax ginseng* and *P. quinquefolium* after one month of culture, in liquid MS medium supplemented with BSAA 10^{-6} M in darkness

	Panax ginseng multiplication rate		*Panax quinquefolium* multiplication rate		Aspect of the roots
	By FW	By number	By FW	By number	
Depilation	12.3 ± 0.9	14.1 ± 2.1	9.8 ± 1.1	11.4 ± 2.5	Thin, white
Rapid cutting	11.9 ± 1.3	13.0 ± 2.2	10.4 ± 0.8	11.6 ± 1.9	Thin, white
Transfer	1.2 ± 0.3		1.3 ± 0.5		Brown

Table 2. Effect of auxins added to liquid MS medium on the multiplication rate (expressed by FW) of roots of *Panax ginseng* and *Panax quinquefolium* (0.4 g.l^{-1} as initial explant) after one month in darkness (n.d.: not determined)

	Panax ginseng	*Panax quinquefolium*
–	1.7 ± 0.3	n.d.
BSAA 10^{-7} M	5.3 ± 1.2	3.7 ± 0.6
BSAA 10^{-6} M	16.2 ± 2.2	12.4 ± 1.7
BSAA 10^{-5} M	3.8 ± 0.7	4.1 ± 0.8
IAA 10^{-7} M	2.1 ± 1.1	2.3 ± 0.9
IAA 10^{-6} M	2.7 ± 0.9	2.5 ± 0.6
IAA 10^{-5} M	1.4 ± 0.5	1.1 ± 0.2
IBA 10^{-7} M	5.2 ± 1.5	4.0 ± 0.8
IBA 10^{-6} M	4.2 ± 1.8	3.8 ± 0.7

thin. In the presence of IAA, there was no formation of new roots, but only an extension of the original roots, which explains the doubling in weight after one month. When IBA (10^{-7} and 10^{-6} M) was used, the results were better but the roots essentially only extended and only in a few cases, new roots were formed.

3.3 *Effect of sugar types*

Fructose and glucose, in place of sucrose, were also tested in MS medium supplemented with BSAA at 10^{-6} M in an attempt to increase the multiplication rate of hairy-like roots of *Panax ginseng* and *Panax quinquefolium* (Table 3). Fructose and glucose somehow increased the FW of the inoculated roots only. Formation of new roots took place only in the presence of sucrose.

Table 3. Effect of sugar types (30 g.l^{-1}) on the multiplication rate (expressed by FW) of roots of *Panax ginseng* and *Panax quinquefolium* (0.4 g.l^{-1}) after one month of culture, in darkness

	Panax ginseng	*Panax quinquefolium*
Sucrose	14.3 ± 1.9	10.9 ± 1.5
Fructose	1.2 ± 0.4	0.9 ± 0.3
Glucose	1.3 ± 0.3	1.0 ± 0.3

3.4 *Effect of material density*

The tests were performed in 250 ml Erlenmeyer flasks containing 100 ml MS medium supplemented with BSAA 10^{-6} M. Various quantities of roots were used (Table 4): 0.04 g ($\pm$30 roots), 0.10 g ($\pm$75 roots) or 0.25 g ($\pm$180 roots).

The multiplication rate was more than 10 fold with 0.04 g of fresh material/100 ml but decreased with the increase of the root quantity. In the last condition, only root elongation occurred (Figure 1).

The bioreactors (1.5 l of medium) were inoculated with 0.4 to 3 g of *Panax quinquefolium* roots and the media were not changed during the 4 weeks of culture. After 4 weeks of culture, there was no more weight increase. As observed in the Erlenmeyer cultures, the multiplication rate was superior when the inoculum was small (assay 1, Table 5) and decreased when the inoculum increased. Therefore, it was lower than in the Erlenmeyer flasks. The formation of new roots was poor; the old roots swelled.

3.5 *Effect of adsorbent compounds*

For industrial root culture in a bioreactor, it seems likely that 0.4 g of roots per liter of medium, determined as the optimal quantity, is too small. This small

Table 4. Effect of root fresh mass cultured in 100 ml MS medium supplemented with BSAA 10^{-6} M during one month, in darkness and effect of the addition of PVP and AC on the multiplication rate (expressed by FW)

	Panax ginseng			*Panax quinquefolium*		
	0.04 g	0.10 g	0.25 g	0.04 g	0.10 g	0.25 g
BSAA 10^{-6} M	11.0 ± 1.4	7.4 ± 0.9	3.2 ± 0.5	10.8 ± 1.5	4.6 ± 0.4	1.7 ± 0.2
+ PVP 0.5 g.l^{-1}	8.8 ± 1.0	6.7 ± 0.8	2.0 ± 0.3	7.5 ± 1.0	3.6 ± 0.5	1.6 ± 0.3
+ AC 1 g.l^{-1}	1.5 ± 0.2	1.1 ± 0.3	1.4 ± 0.2	2.2 ± 0.4	1.3 ± 0.3	1.2 ± 0.1
+ AC 10 g.l^{-1}	1.3 ± 0.3	1.0 ± 0.2	0.9 ± 0.1	2.5 ± 0.2	1.1 ± 0.1	1.1 ± 0.2

Figure 1. Effect of the quantity of roots (1: 0.04 g; 2: 0.10 g; 3: 0.25 g) cultured in 100 ml MS medium supplemented with BSAA 10^{-6} M during one month, in darkness.

Table 5. Production of roots of *Panax quinquefolium* in bioreactor. In the last assay, the medium was renewed every week

Assay	Time (days)	Inoculum (g)	Fresh matter recovered	Multiplication rate
1	30	0.9	6.0	6.61
2	30	1.4	4.1	2.92
3	30	3	3.3	1.10
4	48	0.4	5.1	12.60

quantity was not able to consume totally an element of the medium. We suggest that the roots released substances in the medium that inhibited root growth.

Polyvinylpyrrolidone (PVP, Fluka K30, Buchs, Zwitzerland) and activated charcoal (AC, Vel, Leuven, Belgium), were added to the media to test the effect of adsorbent compounds on the growth of the hairy-like roots of *Panax ginseng* and *Panax quinquefolium* (Table 4). The addition of these compounds, especially charcoal, had a very negative effect.

3.6 *Effect of periodic medium renewal*

In these experiments, to allow an easy renewal of medium, 0.06 g of roots were placed in a gauze bag in 100 ml medium. When the media were changed every week (Table 6, cond. 2), a small increase of the multiplication rate was observed (only 15% for the *Panax quinquefolium* roots). When the same test was performed in a bioreactor (assay 4, Table 5), the multiplication rate was improved (superior to 12).

These weekly medium renewals also allowed us to test when BSAA is necessary during the root multiplication process (Table 6). It appeared that BSAA was necessary during the first 2 weeks (cond. 4); the multiplication rate in this case was better than when BSAA was used only for one week (cond. 5). Moreover, the

Table 6. Effect of medium renewals (assays 2 to 7) on the multiplication rate (expressed by FW) of roots of *Panax ginseng* and *Panax quinquefolium* in a gauze bag (0.06 g) in 100 ml medium (in 250 ml Erlenmeyer flasks). Assay 1: no medium change; BSAA: MS medium supplemented with BSAA 10^{-6} M; 0: MS medium without BSAA

Assay	Week 1	Week 2	Week 3	Week 4	*Panax ginseng*	*Panax quinquefolium*
1	←———BSAA———→				3.5 ± 0.2	2.7 ± 0.2
2	BSAA	BSAA	BSAA	BSAA	4.1 ± 0.1	3.1 ± 0.1
3	BSAA	BSAA	BSAA	0	3.9 ± 0.2	3.1 ± 0.2
4	BSAA	BSAA	0	0	4.6 ± 0.2	3.7 ± 0.2
5	BSAA	0	0	0	3.2 ± 0.3	2.5 ± 0.4
6	BSAA	0	BSAA	0	4.3 ± 0.2	3.5 ± 0.1
7	0	0	0	0	2.2 ± 0.4	1.9 ± 0.3

multiplication rate obtained with BSAA during the first 2 weeks was higher than when BSAA was used for 3 or 4 weeks (cond. 2 and 3). The results obtained when BSAA was present during the weeks 1 and 3 were similar to those of the assay with BSAA present during the first 2 weeks.

4. Discussion

The earlier studies on *Panax quinquefolium* and *Panax ginseng* with root [26], petiole [27] or cotyledonary [20] explants demonstrated that hairy roots can be obtained after infection by *A. rhizogenes* or *A. tumefaciens*. However, the hairy roots required phytohormones in the medium for satisfactory growth [16]. In the present study, using root explants, hairy-like roots were obtained without infection by *Agrobacterium*. This establishment was dependent upon the season and the auxin used in the initiation medium. Hairy-like root cultures were only achieved in December on MS liquid medium containing BSAA 10^{-6} M. BSAA is a synthetic auxin with powerful auxin like activities [5, 7, 18]. The roots were subcultured by transfer every 4 weeks to the same fresh medium after rapid cutting (± 10 cuttings by explant). The multiplication rate recorded in this case (Table 1) was much higher than that obtained after infection by *Agrobacterium* by [26]. Various assays were performed to increase the multiplication rate of the hairy roots (type and level of auxins (Table 2) and of sugar (Table 3)) but the results suggested that the best medium was the first used.

A problem was identified when the density of the hairy roots was increased. 0.4 g of roots per litre was the best quantity in Erlenmeyer flask cultures (Table 4). The same phenomenon was observed in bioreactors (Table 5). Two hypotheses may explain this phenomenon; the first being a depletion of nutrients of the medium. However this is hardly explained by such a low quantity of roots. The second possibility is that the roots secrete toxic substances into the medium. To test this last possibility, two types of assays were performed:

- adsorbents such as polyvinylpyrrolidone or activated charcoal were added to the medium; this treatment was ineffective (Table 4);
- the media were renewed every week. In this case, the multiplication rate increased slowly (Table 6) with more materials but was not comparable with the multiplication rate obtained with a very small quantity of roots. Moreover, the multiplication rate of the roots placed in a gauze bag (for medium change test) was very small comparatively to the same experiments without a bag (3.5 for 0.06 g of *P. ginseng* roots in 100 ml in gauze bag/7.4 for 0.1 g of *P. ginseng* roots in 100 ml without bag). Conversely, the effect of the medium renewals in a bioreactor was clear: the multiplication rate was better when the medium was changed every week. In the Erlenmeyer flasks, the positive effect of medium renewals was probably counteracted by a closeness effect of the roots in the bag.

The poor multiplication rate observed when the quantity of roots was increased leads to hypothesize that a “toxic substance” was rejected in the medium by the roots.

The medium renewal experiments also showed that BSAA was not necessary during the whole culture period. The best results were obtained when BSAA

was present during the first 2 weeks and absent later on. It is known that auxin is necessary for rooting but only during the rooting induction phase [6, 13, 24]. After the induction phase, auxin is no longer required. BSAA is a powerful synthetic auxin [4] used for rooting plants with difficult rooting [12]. In *in vitro* cultures of ginseng, BSAA is also an effective auxin for the establishment and multiplication of hairy-like roots. Its presence is necessary at the beginning of the culture but it may be unnecessary for the second part of the culture. The use of such a synthetic auxin for the initiation of hairy-like roots, in the absence of *Agrobacterium rhizogenes*, is thus a new type of procedure which can be useful for the production of fresh mass and for testing the production of secondary metabolites.

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