0273-2289/04/116/1193–1203/$25.00

Effects of Inoculum Conditions on Growth of Hairy Roots of *Panax ginseng* C.A. Meyer

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Abstract

Plants have a potential to produce a large number of important metabolites such as pharmaceuticals, food additives, pigments, flavors, fragrances, and fine chemicals. Large-scale plant cell and tissue cultures for producing useful products has been considered an attractive alternative to whole plant extraction for obtaining valuable chemicals. In plant cell and tissue cultures, cell growth and metabolite production are influenced by nutritional and environmental conditions as well as physical properties of the culture system. To obtain a high growth rate of plant cell and tissue cultures, the culture conditions should be maintained at an optimum level. We studied the relationship between inoculum conditions and the growth of *Panax ginseng* hairy root culture, and found that the growth rate varied with the inoculum conditions such as the number of root tips, the length of root tips, the part of root tips, and the inoculum size and age of hairy roots.

Index Entries: *Panax ginseng*; transformed hairy root; inoculum condition; inoculum age; root culture.

Introduction

Plants are known as a potential source of a large number of important biochemical constituents *(1–3)*. In recent years, transformed hairy roots have been studied as a potential large-scale source for production of plant-derived useful compounds such as pharmaceuticals. They have several advantages compared to plant cell suspension culture, such as high growth rate, high and stable secondary metabolite productivity, autotrophy of

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plant growth hormones, and inherent genetic stability reflected in stable growth and reproduction *(4,5)*.

Several bioreactor designs have been demonstrated for large-scale culture of hairy roots *(6,7)*. Bioreactors used for hairy root culture are more complex owing to continuous growth of hairy root and must compensate for the heterogeneous, cohesive, structured, and entangled nature of fibrous roots *(4)*. Hairy root cultures may be feasible for large-scale applications, but many problems with the hairy root culture system are still unsolved *(6–9)*.

In plant cell culture, the growth rate is generally affected by inoculum conditions such as cellular age and preculture period *(10)*. For many years, it has been known that inoculum size is a significant factor affecting the performance of plant cell suspension cultures. Inoculum size affects the activities of enzymes in plant cell cultures for a synthesis of secondary metabolites *(11)*. If the inoculum size is sufficiently high, the lag phase may be eliminated or diminished. The root growth morphology may be an important consideration factor in the development of large-scale processes using hairy roots *(12)*. The development of suitable large-scale inoculation techniques and strategies to provide uniform root distribution within bioreactors has met with obstacles *(13)*.

Very few investigations have been carried out on the effect of inoculum conditions on hairy root cultures *(11)*. In recent research of the root culture system, the inoculum studies have often focused on the physical structure and physiologic status of roots *(14)*. Bhadra and Shanks *(15)* reported that the length of individual root tips was an important factor on growth, while the number of tips used in the inoculum had relatively little effect on *Catharanthus roseus* hairy root culture.

Panax ginseng C.A. Meyer, which belongs to the Araliaceae family, is one of the most famous oriental medicinal plants and is found in the Korean peninsula and China. Ginseng plants have many beneficial bioactive effects on human health, such as hemostatic qualities and abilities to promote blood circulation, relieve pain, cure bleeding wounds and trauma, relieve stress, and improve immune functions. The major compounds of pharmaceutical interaction in ginseng have been identified to be saponin (ginsenosides), polysaccharides, antioxidants, peptides, fatty acids, alcohols, vitamins, and phenolic compounds *(16,17)*. The aim of the present work was to investigate the effect of inoculum conditions on the growth and metabolite biosynthesis of *P. ginseng* C.A. Meyer hairy root culture.

Materials and Methods

Hairy Roots and Culture

The hairy roots of *P. ginseng* C.A. Meyer were initiated and maintained as described previously *(18)*. In all experiments, the hairy roots were cultivated in liquid hormone-free 1/2 MS medium *(18)* containing 30 g/L of sucrose. The pH of the medium was adjusted to 5.8 with 2 *N* NaOH, and

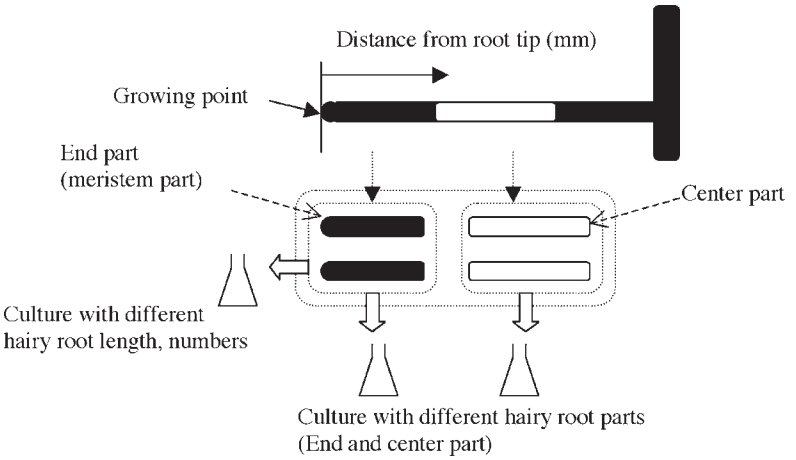

Fig. 1. Schematic of root segment preparation used for inoculum conditions of *P. ginseng* hairy root.

the medium was autoclaved at 121°C for 15 min and cooled to 23°C prior to use. Cultures were incubated at 23°C in the dark in 250-mL Erlenmeyer flask on a rotary shaking incubator (Vision Scientific) operated at 70 rpm.

Experimental Procedure

Shake-flask experiments were carried out to investigate the effect of inoculum conditions on hairy root growth and metabolite biosynthesis. To investigate the effect of the root tip on hairy root growth for 30 d, the end part (meristem part) of the primary root tip was excised about 10 mm from the apical meristem, and the center part was used to 10 mm length after the end part root tips (Fig. 1) *(19)*. Each flask contained 30 mL of medium/100-mL flask and three root tips. To investigate the effect of root tip number and length on hairy root growth, the number of root tips was set at one, three, six, or nine with each 10 mm long, and the root tip length was set at 5–25 mm with three root tips. Each experiment was performed in a 100-mL flask containing 30 mL of medium for 30 d. To determine optimum inoculum age, 5- to 30-d-old cultured roots were used as an inoculant with 1% (w/v) g fresh weight for 30 d in a 250-mL Erlenmeyer flask containing 100 mL of medium. To determine optimum inoculum size in flask culture, 0.2–3.0 % (w/v) inoculum size was tested for 15 d in a 250-mL Erlenmeyer flask containing 100 mL of medium.

For scale-up of inoculum conditions of hairy root cultivation, a 1-L bioreactor (working volume of 800 mL) was used. This bioreactor had a height/diameter aspect ratio of 7.14. The bubble bioreactors had no internal mechanical agitation parts. The supplied aeration rate was 0.1 vvm at the bottom by sparger. Each bioreactor was inoculated with 0.2–2.0 % (w/v) g fresh weight of hairy roots and cultured for 32 d.

Analytical Methods

To determine cell mass, the hairy roots were harvested, rinsed with distilled water, and the extra water was eliminated. Treated hairy roots were measured as fresh weight and dry weight. The dry weight was measured gravimetrically after drying the roots at 60°C for 24 h.

Extraction and Analysis of Crude Saponin and Intracellular Polysaccharide

To determine crude saponin, 100 mg of powdered dry hairy roots was suspended in 5 mL of water-saturated *n*-BuOH, sonicated for 2 h, and centrifuged twice at 5030*g* for 10 min. The collected supernatant was used to determine crude saponin by the vanillin-H_2SO_4 gravimetric methods *(18)*.

To determine intracellular polysaccharide, 100 mg of powdered dry hairy roots was suspended in 10 mL of distilled water, sonicated for 1 h, and centrifuged twice at 5030*g* for 10 min. The collected supernatant was used to determine intracellular polysaccharide by the phenol–sulfuric acid method *(19)*.

Calculation of Growth Rate

Cell growth was evaluated in terms of average cell growth rate (GR). Average growth rate was defined as follow:

GR = (final cell mass – initial cell mass)/initial cell mass/time

The presented data are the average value from three independent experiments each repeated twice.

Results and Discussion

Hairy roots show species- and culture-dependent branching patterns *(20)*. A general characteristic of hairy roots is their prolific branching tendency, resulting in generation of many new root tips and relatively high specific growth rates *(12)*. The physical structure of hairy roots presents handling problems for preparation of inoculum in large-scale cultivation. The management of branching pattern in hairy root cultures is important because branching can influence growth rate and production of metabolites, but branching patterns are not simply related to the inoculum conditions *(20)*. We investigated the effect of inoculum conditions such as inoculum size, root parts, age, and root number on the growth and metabolite production in *P. ginseng* hairy root culture.

Figure 2 shows the effect of the inoculated root tip part on the growth of *P. ginseng* hairy roots. The end part, whose apical meristem of root tip had been excised prior to inoculation, was grown to a total biomass 1.6 times greater than that of the center parts, which are the root tip part after being excised about 10 mm from the end part. In all experiments, new lateral branch formation was observed on the inoculated main root tip.

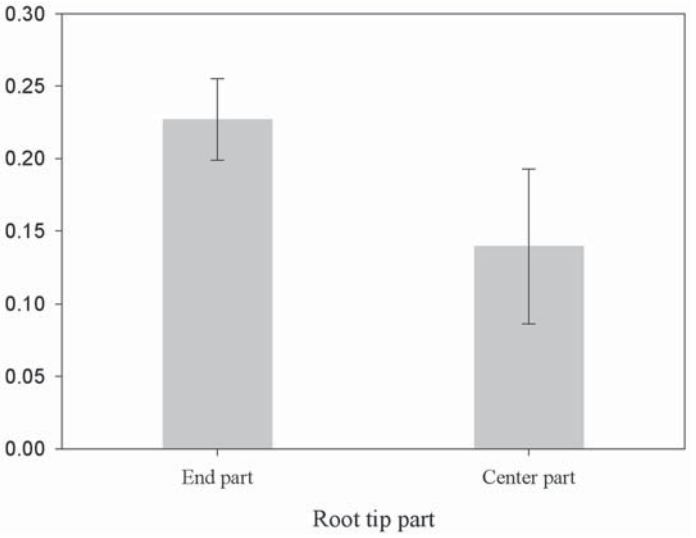

Fig. 2. Effect of inoculated root tip parts on biomass growth in flask culture for 30 d (used root tip length of 5-mm).

The root tip with apical meristem showed length enhancement with lateral root formation. However, the center root tip formed lateral roots without length enhancement of the original root tip (Fig. 3). Some researchers have reported the effect of the apical dominance on growth, which was observed as an increase in specific growth rate for inocula formed by branched roots *(12,14).*

Medium conditioning by increasing cell density is known to be an important factor in plant cell suspension culture *(21).* Increasing the number of inoculated root tips in root culture would be expected to promote more rapid conditioning of the medium and therefore show better growth, but it did not affect the growth in the hairy root experiments reported here. At the beginning of hairy root culture, when the flasks contain only a few root tips, the total oxygen demand is minimal; thus, the mass transfer does not interfere significantly. During the growth period of hairy roots, mass-transfer limitations were not believed to reduce the growth rate of hairy root cultures *(11).* The growth rate depends on the generation of new meristematic growth regions. If the ratio between the number of root tips per length decreases as the root culture grows, the specific growth rate would decline *(14).*

Some suggest the concept that diversity of the hairy root tip—cell division, elongation, and maturation regions—is necessary for optimal growth rate *(15).* The best growth of root tips is consistent with the formation of new branches in maturation zones and enhancement of apical dominance *(14).*

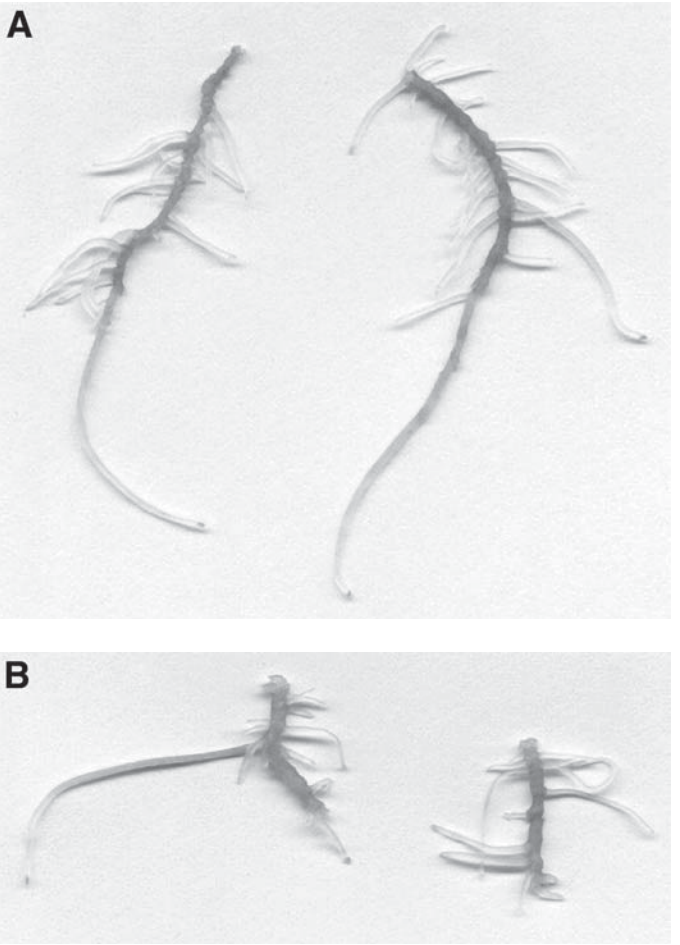

Fig. 3. Growing root segments of *P. ginseng* hairy root in solid culture for 10 d: **(A)** end part; **(B)** center part.

Figure 4 shows the effect of the number of root tips in the inoculum. Increasing the number of root tips from one to six enhanced growth rate. However, the growth rate was reduced by 21% at 9 root tips. These results were caused by several factors, including medium conditioning, mass-transfer resistance, and interactive effect of root tips. The number of inoculated root tips and the initial medium volume had relatively little effect on culture growth for *Catharanthus roseus* hairy roots, and increasing the number of tips from three to nine reduced growth rates by up to 40% in *Atropa belladonna* hairy root culture *(11,15).*

Figure 5 shows the effect of different lengths of root tips in shake-flask cultures. By inoculating shake flasks with root tips of different lengths (5–25 mm), the highest growth rate was obtained for 5-mm-long root tips.

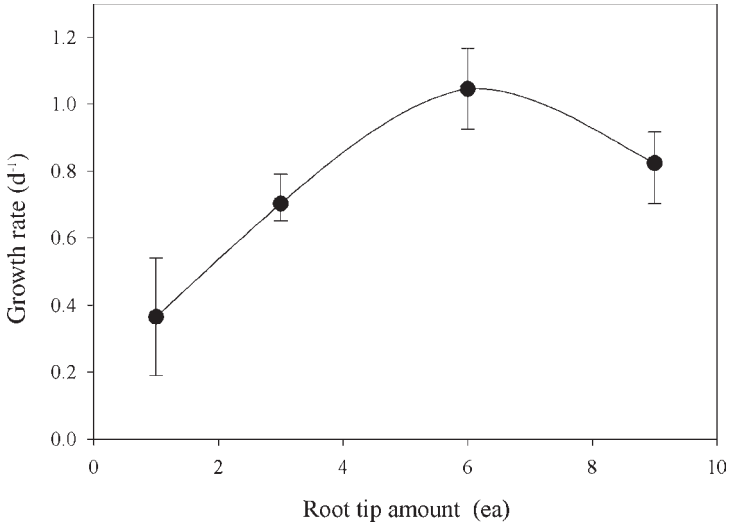

Fig. 4. Effect of inoculated root tip number on biomass growth in flask culture for 30 d.

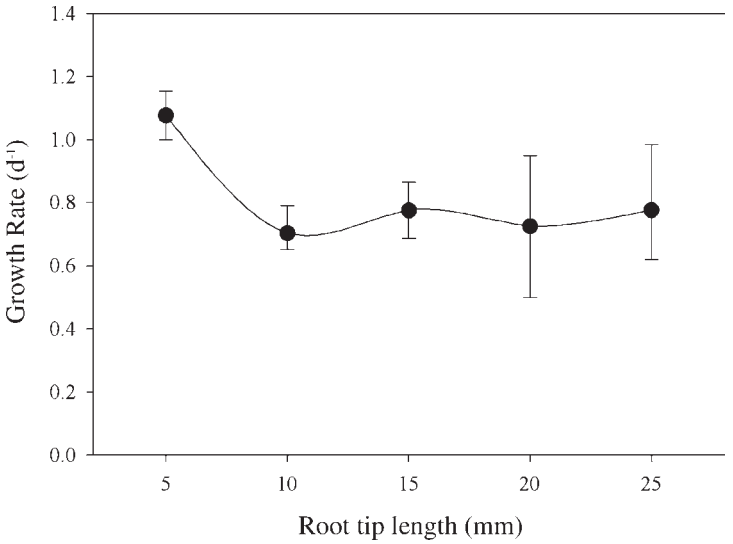

Fig. 5. Effect of inoculated root tip length on biomass growth in flask culture for 30 d (each experiment was performed using three root tips).

Longer tips (10–25 mm) containing apical meristem and maturation zone all had a lower growth rate. We believe that the suppression of growth was caused by a physiologic status of inoculated root tips affected largely by enhancing their apical dominance in spite of retarding the appearance of new lateral branches in the maturation zone. By contrast, Bhadra and Shanks *(15)* have reported that the specific growth rates of culture for 35 to 40-mm-long root tips was 20–60% higher than for cultures with 10 to 15-mm-long root tips in *C. roseus* hairy root culture.

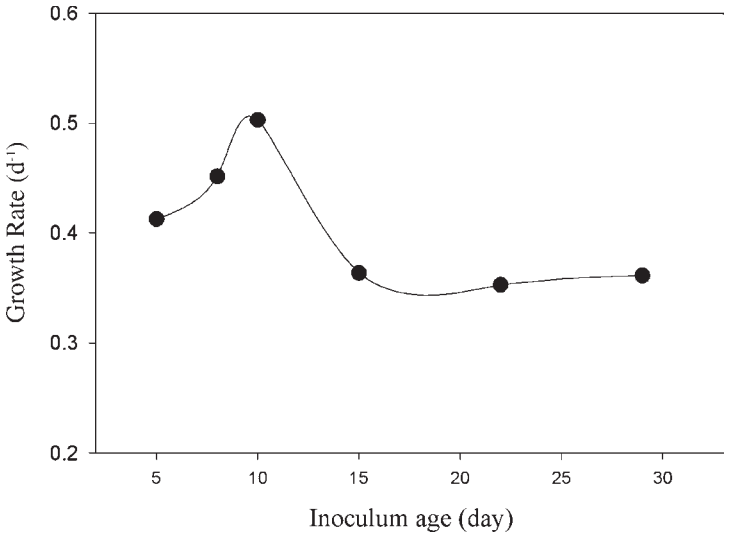

Fig. 6. Effect of inoculum age on biomass growth in flask culture for 30 d.

To investigate the influence of inoculum condition such as precul-tured age of root inoculant, the hairy roots were incubated for various periods (5–30 d) in 250-mL shake flasks. Figure 6 shows the effect of inoculum age on the growth of *P. ginseng* hairy roots with 100 mL of medium in 250-mL flask cultures. Hairy roots inoculated about 1 g fresh weight per 100 mL of medium for this experiment. The maximum growth rate was obtained using inoculum precultured for 10 d in liquid shake-flask cultures. The doubling time of *P. ginseng* hairy roots was about 6.5 d in 1% (w/v) flask cultures *(19),* so the maximum growth rate of hairy roots presented at 8- to 10-d precultured periods. It appears that the higher initial growth rate of precultured inoculum results in shorter lag phase for growth of hairy roots. In *Beta vulgaris* hairy roots, biomass growth was at a maximum in 14-d-old inoculum on solid and liquid medium culture at precultured conditions for 7–21 d *(22).*

Plant cell suspension cultures inoculated at low cell concentrations resulted in a typical growth rate reduction by long lag phase periods, whereas plant root cultures showed an improvement in growth rate. These contrasting growth patterns can be explained as a result of the basic physical and physiologic differences such as the high specific surface area of cells as compared to roots and the differentiated structure of roots *(14).*

Figure 7 shows the effect of inoculum size on growth rate in 250-mL flask cultures. Maximum growth rate occurred with an inoculum size of 0.4% (w/v). Above 0.7% (w/v) growth rate was declined. The final cell growth was not proportional to the increase in inoculum size. This result, caused by the growth rate reduction at higher root tissue concentration,

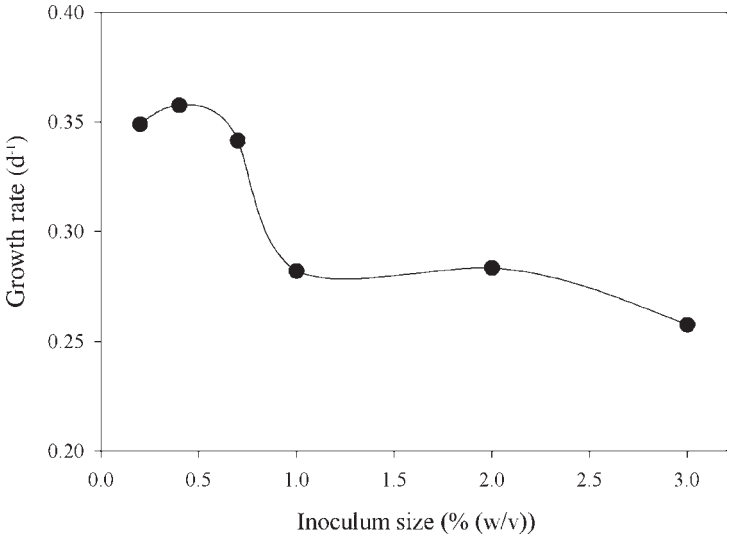

Fig. 7. Comparison of hairy root growth rate on inoculum size in flask culture at 15 d.

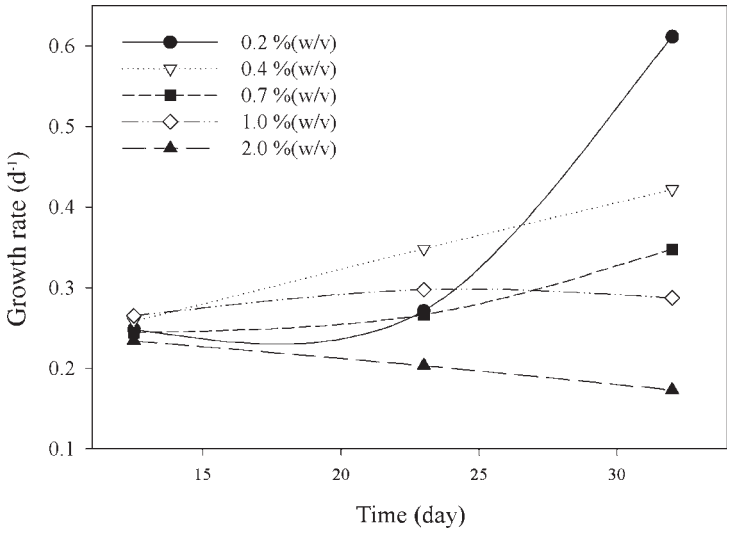

Fig. 8. Effect of inoculum size on biomass growth rate in bioreactor culture for 32 d.

may be owing to the liquid mass-transfer limitations and the depletion of medium nutrients. In root and suspension cultures of *Hyoscyamus muticus*, effective specific growth rates steadily increase as inoculum size decreases, whereas cell suspensions show a marked decline in growth rate *(14)*.

For large-scale culture, inoculum size is one of the most significant factors affecting plant cell/tissue culture systems. Figure 8 shows the time course of the change in growth rate as inoculum size in bioreactor cultures.

Table 1
Comparison of Metabolite Content on Root Tip Parts of *P. ginseng* Hairy Roots

	Crude saponin (%)	Intracellular polysaccharide (g/g)
End part	10.2	0.201
Center part	17.6	0.174
Whole hairy root	16.4	0.191

The growth rate of hairy roots did not greatly differ for an initial growth period of 13 d. At below 1% (w/v) inoculum size, growth rates were enhanced for 34-d culture periods. In particular, a sharp increase in growth rate for long cultivation periods was observed at 0.2% (w/v) inoculum size. By contrast, the declining growth pattern was above 1% (w/v). As inoculum becomes smaller, there are very few hairy roots in large volume of medium in large-scale culture. As a result of the small amount of hairy roots, the influence of the physiologic and physical status of inoculated hairy roots on overall growth rate becomes important. In fact, the higher growth rate at reduced optimum inoculum tends to reduce the time required to reach the final mass concentration *(14).*

The distribution of metabolites in *P. ginseng* hairy roots cultured in shake flasks for 30 d is shown in Table 1. The crude saponin and polysaccharide content were about 16.4% and 0.191 g/g, respectively, in the whole hairy roots. The metabolite contents of different hairy root sections were as follows: meristem part of root tip (below 10 mm), 10.2 and 0.201; maturation part of root tip (10–20 mm), 17.6 and 0.174 on a dry weight basis. These results are owing to the fact that the meristem part has younger periderm and cortex tissue, which is distributed with oil canal, than the maturation part of hairy root. Root hair and lateral root parts of field-cultured *P. ginseng* roots showed higher crude saponin content than main root parts. In the natural *P. ginseng* roots, saponin content is higher in the periderm and cortex than in the xylem. However, the main part on natural roots has a higher polysaccharide content than the lateral root or root hairs *(19).* Ramakrishnan et al. *(13)* reported that for biomass and specific hyoscyamine content, the total amount of hyoscyamine in the whole roots was equal to the sum of the amounts in the tips and mature parts.

Conclusion

We investigated the effect of inoculum conditions such as the part, number, and length of root tips; age of the hairy roots; and size of inoculant on the growth and metabolite biosynthesis of *P. ginseng* C.A. Meyer hairy root culture. The growth of *P. ginseng* hairy roots in shake-flask cultures was found to vary depending on the inoculum conditions. The end part, whose apical meristem of root tip had been excised prior to inoculation,

was grown to a total biomass 1.6 times that of the center parts, which are the root tip parts after excision about 10 mm from the end part. Root tip with apical meristem showed length enhancement with lateral root formation. However, the center root tip formed lateral roots without length enhancement of the original root tip. Increasing the number of inoculated root tips from one to six enhanced growth rates, but the growth rate reduced by 21% at 9 root tips. By inoculating with root tips of different lengths (5–25 mm), the highest growth rate was obtained for 5-mm-long root tips. At precultured age of root inoculant, maximum growth rate was obtained using inoculum precultured for 10 d. For the experiment on the effect of inoculum size on growth rate in 250-mL flask cultures, maximum growth rate at 0.4% (w/v). Above 0.7% (w/v) inoculum size, growth rate declined. The results of the experiment on the change in growth rate as the result of inoculum size in 1-L bioreactor cultures showed that the growth rate of hairy roots did not differ greatly with an initial growth period of 13 d. At below 1% (w/v) inoculum size, growth rates were enhanced for 34-d culture periods. In particular, a sharp increase in growth rate with long cultivation periods was observed at 0.2% (w/v) inoculum size. By contrast, the declining pattern was shown above 1% (w/v).

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