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Adventitious Root Development and Ginsenoside Production in *Panax ginseng*, *Panax quinquefolium* and *Panax japonicum*

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Abstract

This work was carried out to establish adventitious root culture system in three *Panax* species (wild-grown *P. ginseng*, *P. quinquefolium*, and *P. japonicum*) to analyze their ginsenoside productivity. Adventitious roots were induced directly from segments of seedlings after cultured on MS (Murashige andSkoog 1962) solid medium containing 3.0 mg/l IBA. Omission of NH_4NO_3 from the medium greatly enhanced both the frequency of adventitious root formation and number of roots per explants in all the three *Panax* species. However, elongation of post-induced adventitious roots was enhanced on medium with NH_4NO_3. Two-step culture protocol: NH_4NO_3-free medium for first two weeks of culture, followed by NH_4NO_3 containing medium for further 4 weeks, greatly enhanced the fresh weight increase of adventitious roots in all the three ginseng species. The fresh weight of adventitious roots was high in *P. quinquefolium* and low in *P. ginseng*, followed by *P. japonicum* regardless of the composition of medium. Pattern and content of ginsenosides in adventitious roots differed among the three *Panax* species. Total ginsenoside content of adventitious roots in *P. quinquefolium*, *P. ginseng*, and *P. japonicum* was 8.03, 15.7 and 1.2 mg/g dry weight, respectively. Among the three speices, adventitious roots in *P. quinquefolium* produced highamount of ginsenosides. The pattern of ginsenoside fractions between *P. ginseng* and *P. quinquefolium* was similar but the amount of ginsenoside differed between the two. While, in *P. japonicum*, total ginsenoside content was very low and some ginsenosides such as ginsenoside Rb2 and Rf were not detected. Conclusively, we demonstrate that same culture condition was required for induction and elongation of adventitious roots of three ginseng species but growth of adventitious roots and their ginsenoside production were different among them.

Key words: Adventitious roots, ginsenoside, *Panax*, ammonium nitrate

Introduction

Panax ginseng is perennial herbaceous plant of Araliaceae family and distributed in North East Asia. *P. quinquefolium* is an another important ginseng species (American ginseng), which is distributed in North America. *P. japonicum* is available in Japan and Southern area of China. These ginseng have long been used for important medicine to promote quality of life (Ellis and Reddy 2002; Coleman et al. 2003). Immune system modulation, antitress activity, anti-cancer and anti-diabetic activities are the most notable features of ginseng in laboratory and clinical trials (Vogler et al. 1999 Kiefer and Pantuso 2003; Dey et al. 2003 Yun 2003). The representative secondary compound in ginseng species is ginsenoside, called saponin. Ginsenosides in *Panax* species are dammarane type triterpene glycosides, which are constituted with tetracyclic arglicon. Among the species of genus *Panax*, more than 25 different ginsenosides are produced naturally and the content and constitution of each ginsenoside are different among the species (Shibata 2001).was fresh weight formationtion.

Most of wild *P. ginseng* is nearly extinct in Northeast Asia due to over exploitation, thus conservation of wild ginseng germplasm is need of the hour. Ginseng root is expensive because of troublesome cultivation. The cost of the wild-inseng roots is about 20-100-fold higher. Plant cell, tissue and organ culture methods have been explored

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as potentially efficient alternatives for the micropropagation and mass production of ginseng cells and tissues. Cell culture of *P. ginseng* produced by pilot-scale has been applied commercially to various foods and cosmetic sin Japan (Nitto Denko Co.) (Ushiyama 1991). Recently, adventitious roots are produced from wild grown *P. ginseng* by large-scale bioreactor and have been commercialized (Choi et al. 2000; Yu et al. 2002). However, there is little information on root culture of other *Panax* species.

In our previous report (Han et al. 2006), we noticed that NH_4NO_3 very effective for induction of adventitious roots of *P. ginseng*. This work was aimed to establish the adventitious root culture system for other two *Panax* species (*P. quinquefolium*, and *P. japonicum*), and investigated the influence of NH_4NO_3 on induction and elongation of adventitious roots in three *Panax* species and their ginsenoside production.

Materials and Methods

Plant materials

Seeds of wild *P. ginseng* C.A. Meyer were collected from Hambaek mountain of Jeongseon-kun situated in Kangwon province of South Korea. Seeds of wild *P. quinquefolium* L. were collected from mountain of Wisconsin of USA. Seeds of wild *P. japonicum* C.A. Meyer were collected from Nikko national mountain in Japan. Dehisced seeds after moisture-chilling treatment for 6 months were immersed in 70% ethanol for one min, transferred to 1% sodium hypochlorite for 10 min, and rinsed three times with sterilized water. Zygotic embryos were removed from sterilized seeds and cultured on 1/2 MS (Murashige and Skoog 1962) medium with 1% sucrose and solidified with 0.27% gelrite. After two weeks of culture, roots of seedlings were pieced into 1.0 cm segments aseptically, and used as initiate cultures.

Direct adventitious root induction from three ginseng species

Root segments were cultured on MS, 1/2MS, and 1/2MS lacking NH_4NO_3. Each medium contained 3.0 mg/L IBA, 3% (w/v) sucrose and solidified by 0.27% gelrite. The medium was adjusted to pH 5.8 and autoclaved at 120℃ or 15 min. Cultures were kept at 22±1℃ nder dark for 5 weeks. About 30 root segments were cultured per Petri dish and the experiment was repeated three times. After 5 weeks of culture, frequency, number and length of adventitious roots were recorded.

Shake flask culture and two step culture for active growth of adventitious roots

Root tips from adventitious roots of three *Panax* species formed on 1/2 MS solid medium lacking NH_4NO_3 were excised to 10 mm in length and cultured by transferring to 100 ml Erlenmeyer flask containing 30 ml of MS, 1/2MS and 1/2MS liquid medium devoid of NH_4NO_3. Each medium contained 3.0 mg/L IBA and 3% sucrose. About 300 mg of root segments were inoculated per flask. Flasks were agitated by gyratory shaker at 100 rpm. After 6 weeks of culture, increase in fresh weight was recorded. Each treatment consisted of three replicates and the experiment was repeated three times. Culture room was maintained at 22±1℃ nder 20 μmol $m^{-2}S^{-1}$ with white florescent lamp illumination.

In two-step culture, adventitous root segments (300 mg) were cultured in 1/2 MS medium devoid of NH_4NO_3 for first two weeks and then transferred to 1/2 MS medium with NH_4NO_3 for further four weeks. At the same time, root segments were cultured in 1/2 MS medium with NH_4NO_3 for 6 weeks continuously. Culture condition was the same as that maintained for shake flask culture as mentioned above. Increase in fresh weight of adventitious roots was recorded by one-week interval for 6 weeks of culture period.

Ginsenoside analyses by HPLC

Ginsenosides were extracted following the method described by Ando et al. (1971). One gram of milled powder of freeze-dried adventitious roots was soaked in 80% MeOH at 60?. After the liquid was evaporated, the residue was dissolved in H_2O and washed twice, followed by extraction with H_2O-saturated n-butanol. The butanol layer was then evaporated to produce saponin fraction. Each sample was dissolved in EtOH, then filtrated with SepPak C-18 Cartridge (Waters, USA). The HPLC separation was performed on a NovaPak C18 column (4 μm, 3.9 X 150 mm, Waters, USA).

The ratio of water and acetonitrile for the first 10 min and last 25 min were 75:25 and 63:37, respectively. Flow rate of the mobile phase was 1.2 ml/min, and ginsenosides were monitored at a wavelength of 202 nm. Ginsenosides werecompared with the authentic ginsenoside standards purchased from ChromaDex Inc (California, USA). Quantitative analysis was performed on a one-point curve method using external standards of authentic ginsenosides.

Results and Discussion

Direct adventitious root induction among three Panax species

In our previous report, we found that 3.0 mg/l IBA was suitable for the induction of adventitious roots from the root

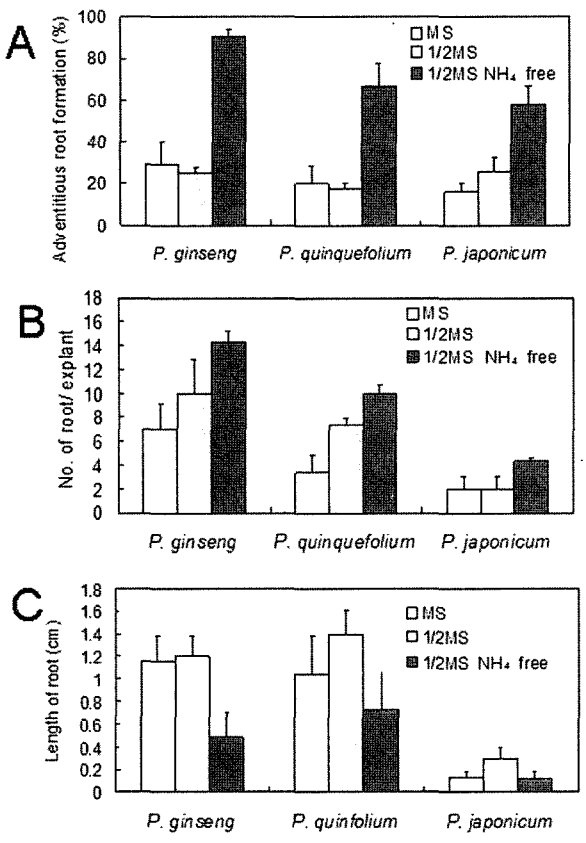

Figure 1. Frequency, number and length of adventitious roots on MS, 1/2MS, and 1/2MS medium devoid of NH_4NO_3 after five weeks of culture from root segments excised from seedlings of *P. ginseng*, *P. quinquefolium* and *P. japonicum*. A: Frequency of adventitious root formation. B: Number of adventitious roots per explants. C: Length of adventitious roots.

segments of wild *P. ginseng* roots (Han et al. 2006). Thus the same auxin was used for the present study. Germinated seedlings of wild *P. ginseng*, *P. quinquefolium* and *P. japonicum* were cut to 10 mm in segments and roots, cotyledons and hypocotyl segments were cultured onto three different solid medium (MS, 1/2 MS, 1/2 MS lacking NH_4NO_3) containing 3.0 mg/L IBA. The excided segments after two weeks of culture produced adventitious roots form their surfaces. Depriving of NH_4NO_3 greatly enhanced the frequency of adventitious root formation and the number of roots per explant as compared to full- or half-strength MS medium with NH_4NO_3 (Fig. 1A,B). Although depriving of NH_4NO_3 stimulated the frequency of adventitious root induction, elongation of post-induced adventitious roots was very slow on 1/2 MS medium lacking NH_4NO_3 as compared to the roots of MS or 1/2MS medium with NH_4NO_3 in all the three species (Fig. 1C). Length of adventitious roots was high on 1/2 MS medium with NH_4NO_3 in all three species (Fig. 1C). This result indicates that lowering or omission of NH_4NO_3 is effective for inducing direct formation of adventitious roots from all three Panaxspecies, however, NH_4NO_3 is necessary for the further growth (elongation) of adventitous roots. In cotyledon culture of *P. ginseng*, depriving of NH_4NO_3 on MS medium produced adventitious roots directly near the surface of excised region of cotyledons but addition of NH_4NO_3 induced somatic embryos instead of adventitious root formation (Choi and Soh 1997). Thus NH_4NO_3 is very important for morphological development in plant tissue culture (Choi and Soh 1997). The direct adventitious root formation by omitting NH_4NO_3 has advantageous for the rapid induction of roots in three *Panax* species.

In our previous report (Han et al. 2006), it was very difficult to induce adventitious roots directly from aged roots of wild *P. ginseng* and the frequency of adventitious root formation was 2.6%. However, explants excised from seedling produced adventitiousroots at 91% directly. The differences in frequency of adventitious root development from seedling and ginseng roots indicates that the morphological potential is highly different depending on the age of plant. The age dependent decline of adventitious root formation may be corresponding to the phenomenon of cyclophysis that vegetative propagation is influenced by age of plants and maturation of the apical meristem (Olesen, 1978)

Shake flask culture and two step culture for active growth of adventitious roots

Liquid culture has more advantages because they are suitable for large-scale culture. Excised segments (300 mg fresh weight) of adventitious roots (Fig. 2A) formed on 1/2 MS solid medium lacking NH_4NO_3 were inoculated into 100 ml Erlenmeyer flasks (MS, 1/2MS, and 1/2 MS lacking NH_4NO_3) with 3 mg/L IBA and 3% sucrose. After 30 days of culture, growth of adventitious roots was assessed. Although induction and elongation of adventitious roots were effective in medium lacking NH_4NO_3 (Fig. 1), total fresh weight increase of adventitious roots was higher in MS and 1/2 MS medium with NH_4NO_3 (Fig. 2E). In *P. quinquefolium*, elongation of adventitious roots was active in MS (Fig. 2B) and 1/2 MS medium (Fig. 2C). Whereas, elongation of adventitious was high suppressedin 1/2 MS mediun lacking NH_4NO_3 (Fig. 2D). Among the media tested, total fresh weight was high in *P. quinquefolium* and low *P. japonicum* (Fig. 2E). Therefore, total fresh weight of adventitious roots was different among the three species regardless of medium type (Fig. 2E).

As induction and elongation of adventitious roots requires different concentration of NH_4NO_3, we designed the two-step culture protocol by changing the NH_4NO_3 of medium during culture time (Fig. 3). Adventitious root segments were cultured in 1/2 MS medium lacking NH_4NO_3 for first two weeks and then the medium was replaced by 1/2 MS medium with original concentration of NH_4NO_3 for further four weeks of culture. Fresh weight and growth of adventitious roots was increased and reached maximum

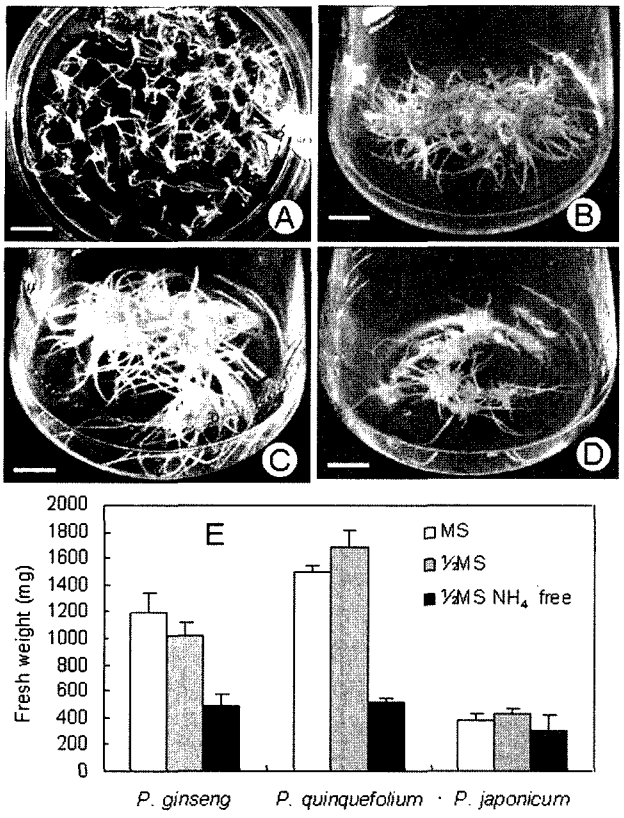

Figure 2. Growth of adventitious roots of *P. ginseng, P. quinquefolium and P. japonicum* in MS, 1/2MS, and 1/2MS medium lacking NH_4NO_3 after 5 weeks of shake flask culture. A: Adventitious roots of *P. quinquefolium* on 1/2 MS medium devoid of NH_4NO_3 after 5 weeks of culture. B-D: Adventitous roots from *P. quinquefolium* in MS medium (B), 1/2 MS medium (C), and 1/2 MS medium devoid of NH_4NO_3 after 5 weeks of culture (D). Bar in A= 15 mm, Bars in B-D= 10 mm. E: Total fresh weight of adventitious roots *P. ginseng, P. quinquefolium and P. japonicum* on MS, 1/2MS, and 1/2MS medium devoid of NH_4NO_3 after five weeks of shake flask culture.

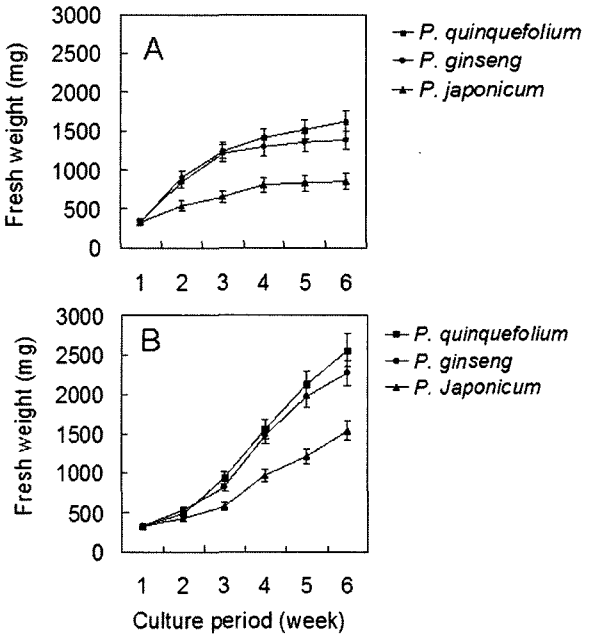

Figure 3. Increase of fresh weight of adventitious roots during 6 weeks of during shake flask culture of *P. ginseng, P. quinquefolium and P. japonicum*. A: Growth of adventitious roots in continuous culture in 1/2 MS medium for 6 weeks. B: Growth of adventitious roots in two-step culture (NH_4NO_3-free medium for first two weeks of culture and NH_4NO_3 containing medium for further 4 weeks).

after 6weeks of culture. This two-step culture technique greatly increased the fresh weight of adventitious roots (Fig. 3). Compared to continuous culture on 1/2 MS medium with or without NH_4NO_3 (Fig. 3A), two-fold increase of fresh weight was noticed by two-step culture (Fig. 3B). In all ginseng species, the two-step culture was effective for enhancing the growth of adventitious roots although growth rate was species dependent manner (Fig. 3). This result indicates that the two-step culture technique by the alternative change of NH_4NO_3 concentration is effective to enhance the production of adventitious roots.

Fresh weight growth of adventitious roots was high in the roots of *P. quiquefolium* and low in *P. japonicum*. In *P. japonicum*, adventitious roots were more slender and showed slow growth compared to the other two ginseng species (data not shown). The differences of adventitious root growth among three *Panax* species on the same culture condition may be due to the genetic make up. The shape of roots in *P. japonicum* is bamboo-rhizome like, thus this species is called to bamboo ginseng. Whereas, both *P. ginseng* and *P. quiquefolium* have similar root morphology. Phylogenetic analysis on the basis of 18S ribosomal RNA gene and *matK* gene sequences of *Panax* species revealed close relationship between *P. ginseng* and *P. quiquefolium*, but not *P. japonicum* (Komatsu et al. 2001).

Comparison of ginsenoside production among three Panax species

Adventitious roots of three *Panax* species produced by two-step culture were sampled. Dried samples of adventitious roots were subjected to ginsenoside analysis by HPLC. Total ginsenoside content was high in P. quinquefolium and low in *P. japonicum* (Fig. 4). When field grown ginseng is compared, *P. quinquefolium* produced higher amount of ginsenosides than that of *P. ginseng* (Washida and Kitanaka 2003). Seven to ten individual ginsenoside profiles between the *P. ginseng* and *P. quinquefolium* was similar (Figs. 4A,B, 5B,C). In both *P. ginseng* and *P. quinquefolium*, ginsenoside Rb1 was high among ginsenosides. While, ginsenoside Re was highest in *P. japonicum* (Fig. 4C). Moreover, in *P. japonicum*, ginsenoside Rb2 and Rf were undetectable (Fig. 4C). Total ginsenoside content of adventitious roots in *P. quinquefolium*, *P. ginseng*, and

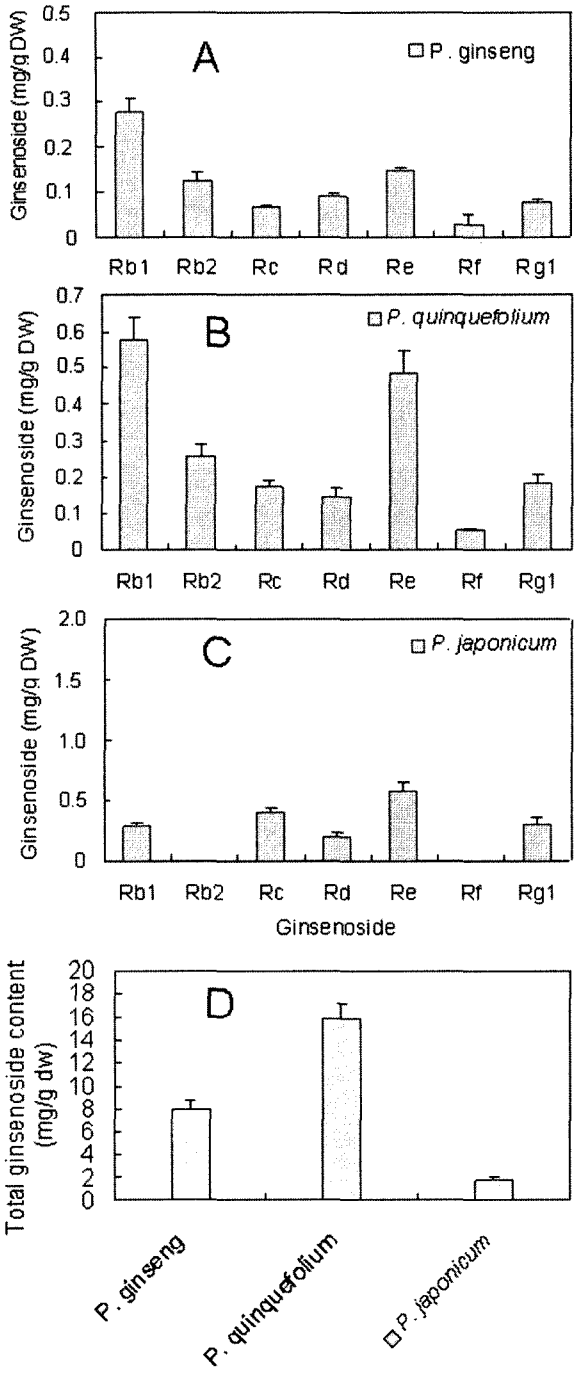

Figure 4. Ginsenoside analysis of adventitious roots in *P. ginseng* (A), *Panax quinquefolium* (B) and *P. japonicum* (C) .

P. japonicum was 8.03, 15.7 and 1.2 mg/g dry weight, respectively (Fig. 4D). In field grown ginseng, there are many differences in ginsenoside profiles in roots between P. japonicum and the other two ginseng species (Zou et al. 2002a,b). In *Panax* japonicum, specific ocotillol-type (chiku-seksusaponins) triterpenes were found to be the major compounds (Zou et al. 2002a).

In conclusion, we noticed that induction, elongation and growth of adventitious roots were regulated by NH_4NO_3 in

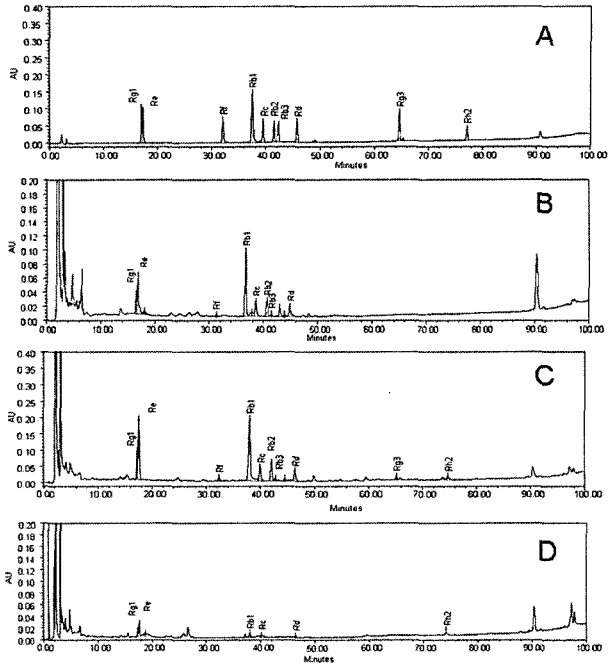

Figure 5. HPLC chromatograms of ginsenosides in adventitious roots. (A), Standard ginsenosides, (B), *P. ginseng*, (C) *Panax quinquefolium* (D) *P. japonicum*.

medium. Generally, growth of adventitious roots and ginsenosides was high in *P. quinquefolium* compared to P. ginseng and *P. japonicum*.

Acknowledgments

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