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Review

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Adventitious Root Cultures of *Panax ginseng* C.V. Meyer and Ginsenoside Production through Large-Scale Bioreactor System

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Abstract

The adventitious root of *Panax ginseng* C.A. Meyer is regarded as an efficient alternative to cell culture or hairy root culture for biomass production due to its fast growth and stable metabolite production. To determine optimal culture conditions for the bioreactor culture of ginseng roots, experiments have been conducted on physical and chemical factors such as bioreactor type, dissolved oxygen, gas supply, aeration, medium type, macro- and micro-elements, medium supplement during culture period, sucrose concentration, osmotic agents, medium pH and light. Elicitation is a key step to increase ginsenoside accumulation in the adventitious roots but biomass growth is severely inhibited by elicitor treatment. To obtain high ginsenoside content with avoiding biomass decrease, we applied two-stage bioreactor culture system. Ginseng adventitious roots were cultured for 40 days to maximize biomass increase followed by elicitation for 7 days to enhance ginsenoside accumulation. We also experimented on types and concentrations of jasmonate to determine optimal elicitation methods. In this paper, we discussed several factors affecting the root propagation and ginsenoside accumulation. Based on the results obtained from previous experiments we have established large-scale bioreactor system (1 ton-10 ton) for the efficient production of ginseng adventitious roots and bioactive compounds including ginsenoside. Still, experiments are on going in our laboratory to determine other bioactive compounds having effects on diet, high blood pressure, DPPH elimination and increasing memories.

Key words: Balloon type bubble bioreactor, bioactive compounds

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Introduction

Ginseng (*Panax ginseng* C.A. Meyer), a member of the *Araliaceae* family, is traditionally considered one of the most potent medicinal plants having anabolic, adaptogenic, antibiotic, minor hyperglycemic, and anti-cancer activities. Ginsenosides have been regarded as the most important active components in ginseng roots. On the other hand, ginseng also has a variety of compounds, e.g., antioxidants, peptides, polysaccharides, fatty acids, alcohols and vitamins (Lee et al. 1995).

The demand for ginseng roots and extracts has been increasing but field production is a long (4 to 6 years) and laborious process, which causes the cultivated roots an expensive commodity (Persons 1995). In addition, disease control practices have led to serious problems with pesticide residues (Yu and Ohh 1995). Likewise, ginseng cannot be replanted in the same land because of various "re-plant diseases", which is a dilemma because Korea has a limited amount of arable land (Li 1995; Yu and Ohh 1995).

In recent years, plant cell culture technology has been successfully applied to the production of many useful secondary metabolites, including pharmaceuticals, pigments, and other fine chemicals (Zhong 1995; Gao et al. 2000). Ginsenosides have also been derived through cell culture (Furuya et al. 1983; Liu and Zhong 1997, 1998; Akalezi et al. 1999) but the high fluctuation in ginsenoside content achieved via culturing is a large obstacle to commercialization. In addition, when ginsenosides have been produced through transformed hairy root cultures (Hwang et al. 1996; Yoshimatsu et al. 1996), the total extracts have contained an opiate-like compound that is potentially harmful to mammalian cells (Yoshikawa and Furuya 1987). Compared with cell culture and hairy root culture, ginseng adventitious root culture has been proven to be an effi-

cient alternative for biomass production due to its fast growth and stable metabolite production. (Seon et al. 1999; Son et al. 1999; Yu et al. 2002).

Callus induction and proliferation of ginseng adventitious roots

Ginseng root pieces were inoculated on MS medium supplemented with 1.0 mg/L 2,4-D, 0.1 mg/L kinetin, and 3% sucrose to induce callus, which were proliferated on MS medium containing 5.0 mg/L IBA and 0.1 mg/L kinetin. Adventitious roots were induced from the callus on MS agar medium supplemented with 2.0 mg/L IBA, 0.1 mg/L kinetin and 3% sucrose. There are distinct stages in adventitious root initiation: a) induction of a new meristematic locus, b) early cell divisions, c) later cell divisions to form an organized and determined root meristem, d) development of the root by cell growth from the meristem. In our experiments, adventitious roots in bioreactors became inflated and looked like callus; perhaps there is a dedifferentiation stage and a redifferentiation stage in the process of proliferation of the adventitious roots. The roots induced were subcultured in 5-liter bioreactors until they reached certain biomass. Adventitious roots of mountain ginseng were also obtained through the same process as in the case of ginseng. 2,4-D (2.0 mg/L) was used for callus induction and IBA for adventitious roots initiation and proliferation (Son et al. 1999 b). Furuya et al. (1983a) found that 2,4-D was essential for callus growth of *Panax ginseng*, but high concentrations of 2,4-D (0.5 mg/L or more) remarkably inhibited the growth. We have concluded that 2,4-D was suitable for induction and growth of the callus, while IBA is favorable for induction and proliferation of the adventitious root in ginseng culture. The procedures of callus and adventitious root cultures are shown in Figure 1.

Bioreactor culture of ginseng roots

Bioreactor culture is the key step towards commercial production of secondary metabolites by plant biotechnology. Bioreactors with computer control systems offer theoretically various advantages over conventional culture procedures due to possibilities of automation, saving labor and reduction of production costs (Preil 1991). Since microbial fermentation techniques were first used in studies on growth kinetics of higher plant cell suspensions (Tulecke and Nickell 1959; Nickell and Tulecke 1960), major progress has occurred in the area of large scale liquid culture and in the development of bioreactor process control system (Fowler 1987; Fowler et al. 1987). Strategies for large-scale production of somatic embryos in suspension culture are preferentially based on the results from *Daucus* (Ammirato and Styer 1985; Styer 1985). Since then bioreactor system was applied for embryogenic and organogenic cultures of several plant species (Levin et al. 1988; Preil et al. 1988; Stuart et al. 1987) and Rittershaus et al. (1989) developed a large-scale bioreactor culture system. In our laboratory, a series of experiments were conducted to establish an efficient ginseng adventitious root growth and ginsenoside production in liquid media (Son and Paek 2001; Yu et al. 2001) and subsequently we established a pilot scale culture of multiple adventitious roots induced from callus using a balloon type bubble bioreactor (BTBB) system (Choi et al. 2001; Yu et al. 2001). Based on the previous results, we have started industrial scale cultures of ginseng adventitious root using 500 to 1000-liter BTBBs (Figure 2) and 10,000-liter BTBBs are under construction.

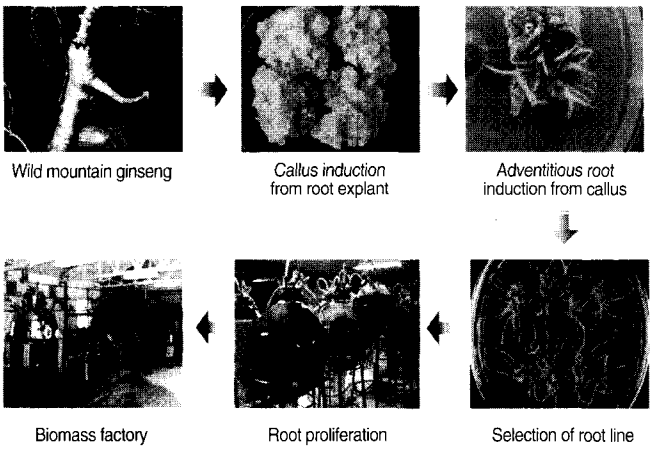

Figure 1. Procedure of ginseng adventitious root culture in large-scale bioreactors.

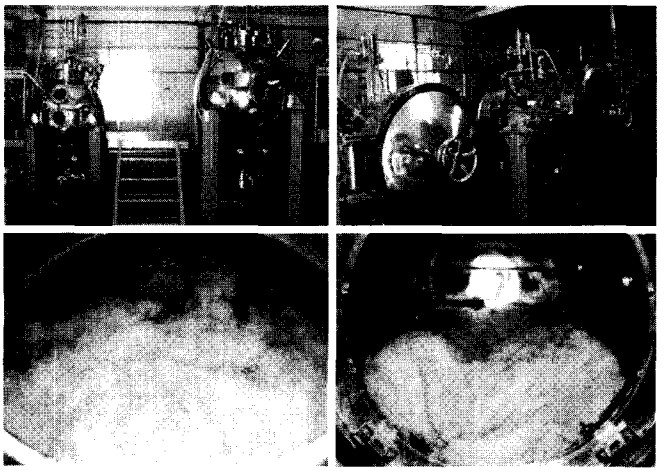

Figure 2. Adventitious roots of mountain ginseng cultured in 500 and 1000 liter bioreactors.

Factors affecting ginseng adventitious root cultures in bioreactors

Medium type and macro- and micro- elements

MS medium was suitable for the root growth but 1/2 or 3/4 MS medium was effective for ginsenoside production. Unlike root growth, ginsenoside accumulation was prevented by NH^{4+} in the culture medium and low concentrations of K^+, Mg^{2+}, and Ca^{2+} were effective for ginsenoside production. In addition, ginsensoide accumulation increased with increasing $CuSO_4$, $ZnSO_4$, and $MnSO_4$ concentrations in the culture medium. However, KI and $CoCl_2$ inhibited both the adventitious root growth and ginsenoside production when they were contained in the medium at high concentrations. Therefore, KI and $COCl_2$ concentrations should be controlled at low levels in ginseng adventitious root culture.

In the investigations of the root growth and ginsenoside accumulation as influenced by NH_4^-/NO_3^- ratio in MS medium, NO_3^- played more important role in biomass increase and ginsenoside accumulation rather than NH_4^+. Maximum ginsenoside productivity was obtained when nitrate was used as the sole N source and the greatest adventitious root biomass was obtained at a 1:2 ratio of NH_4^+ to NO_3^-, which also resulted in high ginsenoside productivity. Similar results were obtained in *Panax quinquefolium* cell culture (Zhong and Wang 1998). They reported that a high growth rate and a great final cell biomass were obtained at low ratios of ammonium to nitrate, and the maximum production of saponin and polysaccharide was archived when nitrate was used as the sole nitrogen source. The maximum production of ginseng saponin and polysaccharide was obtained at 0:1 and 1:2 ammonium/nitrate ratios, respectively. Kaul and Hoffman (1993) reported that high concentrations of ammonium inhibited callus and cell growth in *Pinus strobus* cell culture. In cell culture of *Lithospermum erythrorhizon*, shikonin synthesis was inhibited by ammonium ions in the culture medium and the addition of nitrate was required at the end of the growth phase (Tabata and Fujita 1985).

As described above, nitrate and ammonium ions have different effects on cell cultures, as well as in tissue and organ cultures. Nitrate is known to promote secondary metabolite synthesis, while ammonium inhibits it. Therefore, it is very important to select optimal nitrogen sources and ammonium/ nitrate ratios according to plant species in the secondary metabolite production.

Medium supplement

Adventitious root growth increased double in six weeks when the same amount of the initial medium was supplemented after 3 weeks of culture. Sucrose content and electric conductivity decreased right after feeding the medium, while fructose and glucose contents increased. All anion contents (Cl^-, NO_3^-, $H_2PO_4^-$, SO_4^-) decreased after the medium supplement. In case of cations, NH_4^+ was almost depleted in 3 weeks of culture before feeding the medium and was consumed all after feeding the medium, while there were little changes in Na^+ and K^+ during the whole culture period. Mg^{2+} and Ca^{2+} were absorbed gradually as in the case of anions. These results indicate that sucrose and NH_4^+ are the key elements for ginseng adventitious root growth.

Sucrose concentration

Sucrose is an important carbon source for plant cell and tissue cultures. It has been demonstrated that initial sucrose concentration can affect a number of culture parameters such as growth rate and yield of secondary metabolite in plant cell cultures. For example, in the cell suspension culture of *Perilla frutescens*, a relatively higher sucrose concentration (45 g/L) was favorable to anthocyanin production (Zhong and Yoshida 1995). Zhang et al. (1996) reported that the initial sucrose concentration affected cell growth and saponin production in Panax ginseng. They obtained the maximum cell growth at an initial sucrose concentration of 30 g/L and the maximum saponin production at initial sucrose concentrations of 6 to 8 g/L. The results of our experiment also confirmed the importance of initial sucrose concentrations on the adventitious root growth of *Panax ginseng*. Among sucrose concentrations of 10, 20, 30, 50, 70, and 90 g/L, Root dry weight increased the most at 50 g/L, showing maximum fresh weight, dry weight, and growth rate. However the root growth started to decrease from the sucrose concentration above 70 g/L. The effect of sucrose concentration on ginsenoside production was not as significant as in the case of biomass increase.

Osmotic agents

Sorbitol and mannitol also affected the root dry weight and the ginsenoside accumulation. The root growth was maximized at 0.1 M mannitol or sorbitol but ginsenoside content lowered. Especially, a high concentration (0.5 M) of mannitol strongly inhibited ginsenoside accumulation. Further experiments are required to select optimal osmotic agent and concentration for both root growth and ginsenoside production.

Medium pH

In plant tissue culture, pH is an important factor influencing biomasss increase and secondary metabolite accumulation. In

suspension cultures of red bean, *Vigan angularis*, the development of phenylalanine ammonia lyase (PLA) activity and the accompanying accumulation of isoflavone glucosides were accelerated by merely raising the pH of the medium (Hattori and Ohta 1985). In ginseng root cultures, the initial medium pH should be controlled in the range of 6.0-6.5 before autoclaving since the root growth and ginsenoside content were greatest at the pH range of 6.0 to 6.5. Root growth and ginsenoside accumulation were strongly inhibited when the initial pH was maintained below 4.0 or above 7.0. On the other hand, it is unclear how increasing pH affects enzyme activity during cell and tissue cultures.

Light

The characteristics of light such as PPF, spectral quality, and photoperiod have all been known to influence plant tissue cultures. With respect to production of secondary metabolites, stimulatory effect of darkness was confirmed in the production of betacyanin and polyphenols whereas continuous illumination strongly suppressed the formation of anthracene derivatives (Berlin et al. 1988; Davies 1972). In case of ginseng culture, light stimulated synthesis of ginsenosides and resulted in higher ginsenoside productivity compared with darkness. Alterations of the synthesis pattern of secondary metabolites were reported in light- and dark-grown cultures of several species. Drapeau et al. (1987) demon-strated a shift from ajmalicine accumulation to serpentine accumulation and the alteration of paniculides accumulation under continuous light. Flavonoid and anthocyanins were not produced without light irradiation. In many cases, light triggered biosyn-thesis of second metabolites in plant cell and tissue cultures (Kreuzaler and Hahlbrock 1973; Mantell and Smith 1983).

Ginsenoside accumulation by jasmonate treatments

Jasmonic acid and its derivatives are considered to be involved in a part of the signal transduction pathway that induces particular enzymes catalyzing biochemical reactions to form defense compounds of low molecular weight in plants, such as polyphenols, alkaloids, quinones, terpenoids, and polypeptides (Kushiro et al. 1997; Lee et al. 1995). In *Lithospermum* cell cultures, jasmonates caused a rapid increase in the activities of enzymes involved in the biosynthesis of shikonin such as p-hydroxybenzoate geranyltransferase (Levin et al. 1988). Ginsenosides belong to triterpenoide saponins. They are originated from acetyl-Co A through more than 15 metabolic steps, some of which are not clearly known (Li 1995). Among ginsenosides, Rb1, Rb2, Rc, and Rd belong to Rb group ginsenosides (protopanaxadiols), while Re, Rg1, and Rf belong to Rg group ginsenosides (protopanaxatriols) (Liu and Zhong 1998). Most ginsenosides possess a dammarene skeleton in their aglycone moiety. Aglycones of two major ginsenosides, ginsenoside Rb and ginsenoside Rg, are protopanaxadiol and protopanaxatriol, respectively, which are derived from dammarenediol by hydroxylations (Mantel and Smith 1983). Until now, enzymes related to the synthesis of protopanaxadiol and proto-panaxatriol ginsenosides have not been determined.

Two stage bioreactor culture for biomass increase and ginsenoside accumulation

Five kinds of jasmonate were used for elicitation: Methyl dihydro jasmonate (MDJ), methyl epi-jasmonate (MEJ), methyl epi-dihydro jasmonate (MEDJ), jasmonic acid (JA) and methyl jasmonate (MeJA). Ginsenoside content increased in all treatments regardless of concentrations. Especially, MEJ resulted in the highest ginsenoside accumulation but severely inhibited the root growth. Fresh weight, dry weight, and growth ratio of the roots decreased with increasing jasmonate treatments. To solve the problem, two-stage culture was applied. Root growth was maximized for 40 days followed by jasmonate treatment for 7 days to increase ginsenoside accumulation. Compared with control, total ginsenoside content increased 5-7 folds after jasmonate treatment. Ginsenoside accumulation was also affected by medium composition during elicitation. The effect of elicitation was greatly enhanced when conducted in used medium or distilled water, while the effect was not significant when the elicitation was done in fresh medium.

Accumulation of ginsenosides after methyl jasmonate treatment

Among jasmonates, methyl jasmonate was selected for elicitation due to higher effect and lower price compared to others. The contents of total ginsenoside and Rb group ginsenosides gradually increased and reached the maximum value in 7 days after treatment and showed little changes thereafter. Ginsenosides mainly increased were Rb group, while the contents of Rg group ginsenosides kept stable. Among ginsenosides, the contents of Rgb1, Rc, Rb2, and Rd increased more than those of other groups. In our experiment, methyl jasmonate promoted specifically the accumulation of protopanaxadiol ginsenosides (Rb) rather than the accumulation of protopanaxatriol ginsenosides (Rg) (Yu et al. 2002). This result suggests that methyl jasmonate might have triggered the enzyme activities for the synthesis of protopanaxa-diol ginsenosides. Further

work will be required to investigate the related enzymes involved in the enzymatic biosynthesis of ginsenosides.

Conclusion

Bioreactor system has many advantages of biomass increase and secondary metabolite production. Production can be focused on a desirable form such as cells, organs or plantlets, and the production process can be conducted in a factory setting by automation. Consequently, production period and cost are substantially reduced, product quality is controlled and standardized, products are free of pesticide contamination, and production is conducted all year round. Elicitor-induced accumulation of secondary metabolites has received much attention during the past decade. In case of ginseng, we have established effective elicitation methods to increase ginsenoside content through results from various experiments. Still, experiments are on going in our laboratory to determine other bioactive compounds having effects on diet, high blood pressure, DPPH elimination and increasing memories.

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