0273-2289/05/121–124/1147–1158/$30.00

Production of Antioxidant Compounds by Culture of *Panax ginseng* C.A. Meyer Hairy Roots

I. Enhanced Production of Secondary Metabolite in Hairy Root Cultures by Elicitation

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Abstract

Ginseng (*Panax ginseng* C.A. Meyer) hairy root cultures, established by infecting ginseng root discs with *Agrobacterium rhizogenes*, were used for secondary metabolite production. In this study, several elicitors [salicylic acid (SA), acetylsalicylic acid (ASA), yeast elicitor, and bacterial elicitor] were used to improve the productivity of useful metabolite in *P. ginseng* hairy root cultures. In SA elicitation, total ginseng saponin content increased slightly at lower elicitor dosages (0.1 to 0.5 m*M*). Also, the use of ASA as an elicitor resulted in the inhibition of biomass growth and an increase in total ginseng saponin content at every elicitor dosage (0.1 to 1.0 m*M*) by about 1.1 times. With yeast elicitor addition, hairy root growth was inhibited about 0.8-fold on a dry weight basis compared to the control, but total ginseng saponin content increased by about 1.17 times when compared to the control. The bacterial elicitor showed a slight inhibition of biomass growth, but total ginseng saponin content increased by about 1.23 times upon the addition of 1 mL.

Index Entries: *Panax ginseng*; transformed hairy roots; elicitation; yeast elicitor; ginseng saponin.

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Introduction

Plants are known to be a potential source of a large number of important biochemical constituents (*1*,*2*). Hairy roots induced by *Agrobacterium rhizogenes* also serve as a valuable source of root-derived phytochemicals that are useful as pharmaceuticals, cosmetics, pigments, and food additives. These hairy roots can also synthesize more than a single metabolite and therefore may prove to be economical for commercial production purposes. Transformed hairy roots of many plant species have been widely studied for the in vitro production of secondary metabolites. Transformed root lines can be a promising source for the constant and standardized production of useful metabolites from plants. Also, hairy root cultures follow a definite growth pattern, and so metabolite production may not be related to hairy root growth (*3*,*4*).

Plants produce secondary metabolites in nature as a defense mechanism against attack by pathogens. Plants have been found to elicit the same response as the pathogen itself when challenged by compounds of pathogenic origin. Elicitors are signals triggering the formation of secondary metabolites (*5*). A wide variety of elicitors have been employed to modify cell metabolism in order to improve the productivity of useful metabolites in plant cell and tissue cultures (*6*).

Elicitors are compounds or treatments that induce plants to synthesize phytoalexins at elevated levels. Biotic and abiotic elicitors are used to stimulate secondary metabolite product formation in plant cell/tissue cultures, thereby reducing the processing time needed to achieve high product concentrations (*5*). Elicitors of non-biological origin (abiotic elicitors), such as heavy metals and ultraviolet light, which induce phytoalexin synthesis, are usually termed abiotic stresses to distinguish them from elicitors of biological origin (biotic elicitors), such as polysaccharides, glycoproteins, enzymes, and chitosan derived from fungal, bacterial, and yeast. These compounds were reported for the production of various secondary metabolites (*5*,*7*). The effect of the elicitors depend on many factors, such as the treated concentration of elicitor, the growth stage of the culture at the time of elicitation, the period of contact and the time course of elicitation (*7*).

The active mechanisms of biotic and abiotic elicitors are considered to be different and complex. Because little is known about the biosynthetic pathways of most secondary metabolites from plants, the effect of elicitors on the plant cell/tissue culture cannot easily be predicted. Therefore, elicitation approaches are performed by trial and error (*7*).

Some investigations have been carried out on the effect of elicitation on the hairy root cultures. In recent research of the root culture system, the elicitation studies have often focused on the formation of secondary metabolites and physiological status of roots (*8*,*9*).

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. The major compounds of pharmaceutical interaction in ginseng have been identified to be ginseng saponin, polysaccharides, and phenolic compounds (*3*). The aim of this work is to investigate the effect of several elicitors on the growth and metabolite biosynthesis of *P. ginseng* C.A. Meyer hairy root culture.

Materials and Methods

Hairy Roots Culture and Maintenance

The hairy roots of *P. ginseng* C.A. Meyer were initiated and maintained as described previously (*3*). In all experiments, the hairy roots were cultivated in liquid hormone-free 1/2 MS medium (*3*) containing 30 g/L sucrose. The pH of the medium was adjusted to 5.8 with 2 *N* NaOH, and the medium was autoclaved at 121(C for 15 min and cooled to 23±1ºC prior to use. Cultures were incubated at 23±1ºC in the dark in a 250 mL Erlenmeyer flask on a rotary shaking incubator (Vision Scientific, Ltd.) operated at 80 rpm.

Experimental Procedure

Elicitation by Salicylic acid (SA) and Acetylsalicylic acid (ASA)

Preparation of SA and ASA

SA and ASA were prepared as concentrated solutions and the pH adjusted to 5.8 with 1 *N* KOH before autoclaving at 121ºC for 15 min.

Effect of SA and ASA on the Growth of Hairy Roots

P. ginseng hairy roots (1 g fresh weight) were inoculated into 250 mL Erlyermeyer flasks containing 100 mL phytohormone-free 1/2 MS liquid medium supplemented with 3% (w/v) sucrose, and different concentrations of SA (0 to 10 m*M*) and ASA (0 to 0.5 m*M*). Each treatment was repeated twice.

SA and ASA Dosage Response

Hairy roots were subcultured with an inoculum of about 1 g fresh weight root segments in 100 mL of hormone-free 1/2 MS liquid medium containing 30 g/L sucrose in 250 mL flasks. Cultivation was performed on an orbital shaker at 80 rpm in darkness at 23±1ºC. After 21 d of cultivation, different concentrations of SA (0–1.0 m*M*) and ASA (0–1.0 m*M*) solution or 1 mL of distilled water (control) was added in the culture medium. On d 3 of elicitation, hairy roots were harvested and the biomass and metabolite content were determined. The experiment was performed in triplicate.

Elicitation by Yeast Elicitor

Preparation of Yeast Elicitor and Dosage Response

A carbohydrate fraction isolated from the yeast extract was prepared by ethanol precipitation. Briefly, 50 g of the yeast extract was dissolved in

250 mL of distilled water. Ethanol was added to 80% (v/v). The precipitate was allowed to settle for 3 d at 5ºC and the supernatant was decanted and discarded. The gummy precipitate was dissolved in 250 mL of distilled water. The ethanol precipitation was repeated. The second ethanol precipitate was dissolved in 200 mL of distilled water, yielding a crude preparation that was used without further purification.

The culture method and the medium were the same as those used in the SA experiment. After 21 d of cultivation, different concentrations of yeast elicitor solution were added to the culture medium. On d 3 of elicitation, hairy roots were harvested to determine the biomass and metabolite content.

Elicitation by Bacterial Elicitor

Preparation of Bacterial Elicitor and Dosage Response

Agrobacterium rhizogenes (KCTC 2744) was used as bacterial elicitor. *A. rhizogenes* was grown in 300 mL flasks containing 100 mL of YEB broth in a shaking incubator (120 rpm, 30ºC) for 3 d. The harvested cell was concentrated with a saline solution and diluted to a cell concentration of about 1.0 g/L. Treated cell solution was used as bacterial elicitor without any other treatment. The culture method and the medium were the same as those used in the SA experiment.

Analytical Methods

To determine cell mass, the hairy roots were harvested, and rinsed with distilled water, and the extra water was discarded. 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 hr.

Extraction and Analysis of Total Ginseng Saponin

To determine total ginseng saponin, 100 mg of powdered dry hairy roots was soaked in 5 mL *n*-BuOH saturated with distilled water, stored at 4ºC for 24 hr, sonicated in an ultrasonic cleaning bath for 60 min, and centrifuged twice at 10,000 rpm for 10 min. Collected supernatant was used for total ginseng saponin analysis. Total ginseng saponin was measured by vanillin–H_2SO_4 colorimetric method.

The method was based on a color reaction of the acid-hydrolysis products of the ginseng saponin with vanillin. A 200 (L sample, 0.5 mL of 5% vanillin in EtOH and 5 mL 72% sulfuric acid, were added to a test tube and mixed for reaction. The test tube was placed in a water bath at 60ºC for 10 min. After the reaction was finished, the test tube was cooled in cooled water, vigorously vortexed, and used for detecting absorbance. The absorbance of the product was read at 540 nm using a spectrophotometer (DR/4800, HACH, USA) against a calibration curve established with a ginsenoside Re standard. Authentic ginsenoside Re was purchased from Sigma-Aldrich Co., Ltd.

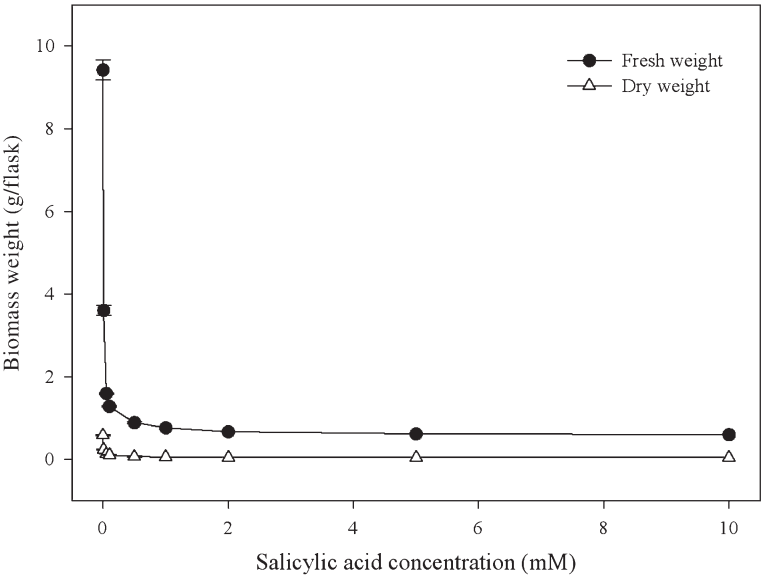

Fig. 1. Effect of salicylic acid on growth of hairy roots.

Results and Discussion

Effect of Salicylic Acid on Growth and Secondary Metabolite Production

Salicylic acid (SA) is a naturally occurring phenolic compound found in many plants. SA supplied exogenously affects various plant physiological and biochemical processes, and acts as a signaling molecule in plant disease resistance, flowering, and thermogenesis. SA has been reported to enhance the productivity of some metabolites in plant cell/tissue cultures (*10*). The occurrence of SA at a high concentration in plants affects growth, as is known in the case of *Vicia faba*, where treatment with SA higher than 3.5 m*M* considerably decreases the rate of root growth (*11*).

SA had obvious effects on both growth and secondary metabolite production. SA strongly inhibited hairy root growth as shown in Fig. 1. The growth of hairy roots decreased with SA concentration in the tested range (0.01–10 m*M*). Also, SA inhibited secondary metabolite accumulation in hairy roots. Upon the addition of 0.01 m*M* SA, total ginseng saponin content was 50.9±4.9 mg/g (0.84 times that of the control).

In a suspension culture of *Hyoscyamus muticus* treated with 40 μ*M* SA, production of lubimin increased by 50%, while in a transformed root culture of the same species, solavertivone production increased by 48% with the addition of 4 μ*M* SA (*12*).

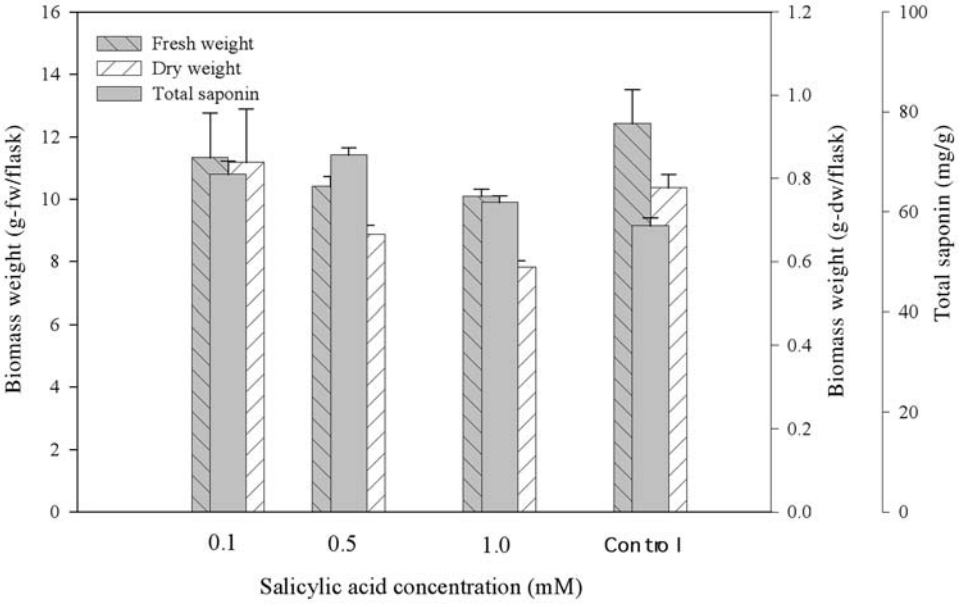

Fig. 2. Effect of salicylic acid on metabolite accumulation of hairy roots.

Effect of SA Elicitor Dosage

As shown in Fig. 2, the use of SA at all four concentrations resulted in inhibition of biomass growth [to as much as 12.43 g-fw/flask (0.778 g-dw/flask) compared with 11.33 g-fw/flask (0.839 g-dw/flask) in the control with the addition of 0.1 m*M* SA]. Total ginseng saponin content at 0.5 m*M* SA elicitation increased over the control (as much as 71.4 mg/g of dry weight compared with 57.2±1.6 mg/g in the control). In SA elicitor dosage experiments, the content of total ginseng saponin increased slightly at lower elicitor dosages (0.1–0.5 m*M*).

Effect of Acetylsalicylic Acid on Growth and Secondary Metabolite Production

Acetylsalicylic acid (ASA, chemical derivates of SA) has been reported to enhance the productivity of some metabolites in plant cell/tissue cultures (*10*).

ASA showed obvious effects on growth of hairy roots and secondary metabolite production. ASA strongly inhibited hairy root growth at low concentrations as shown in Fig. 3. After 21 d with the addition of 0.01 m*M* SA, total ginseng saponin content was 58.2 mg/g (0.96 times that of the control).

Effect of ASA Elicitor Dosage

As shown in Fig. 4, the use of ASA at all four concentrations resulted in the inhibition of biomass growth [to as much as 8.3±0.8 g-fw/flask

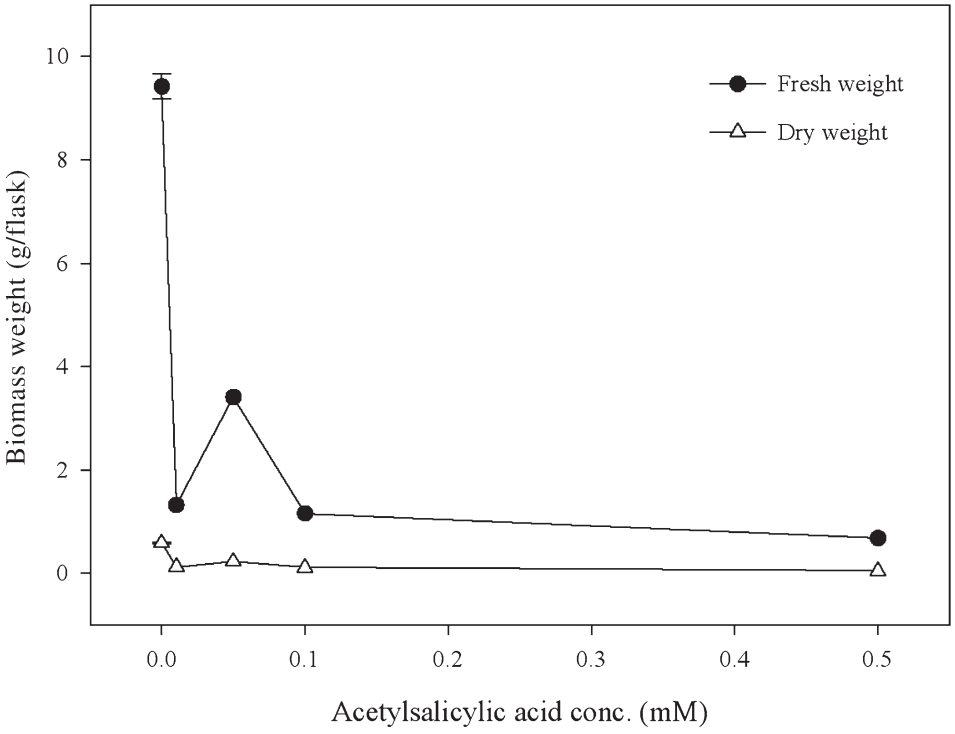

Fig. 3. Effect of acetylsalicylic acid on growth of hairy roots.

(0.42±0.05 g-dw/flask) compared with control (12.4±1.0 g-fw/flask (0.778±0.03 g-dw/flask)) with 1.0 m*M* ASA addition]. Total ginseng saponin content increased at every elicitor dosage (0.1 to 1.0 m*M*) (to as much as 52.4±0.69 mg/g of dry wt compared with 57.2±1.6 mg/g in the control). Total ginseng saponin accumulation by ASA was maximized with 0.5 m*M* ASA addition. Metabolite productivity (biomass weight (metabolite content) was, on the other hand, maximized with 0.1 m*M* ASA addition.

Gregori and Victor (*13*) reported that the addition of various concentrations (0.5–20 m*M*) of ASA to tumor lines of *Cath. roseus* cultured *in vitro* produced remarkable effects on secondary metabolite production.

Effect of Yeast Elicitor on Growth and Secondary Metabolite Production

As shown in Fig. 5, the use of yeast elicitor at all three concentrations resulted in a great decrease in biomass growth to as much as 8.68±0.76 g-fw/flask (0.625±0.03 g-dw/flask) compared with 12.43±1.0 g-fw/flask (0.80 g-dw/flask) in the control. With the addition of 1 mL yeast elicitor, hairy root growth was inhibited by about 0.7-fold on a fresh weight basis (0.8-fold on a dry weight basis) relatively to the control and,

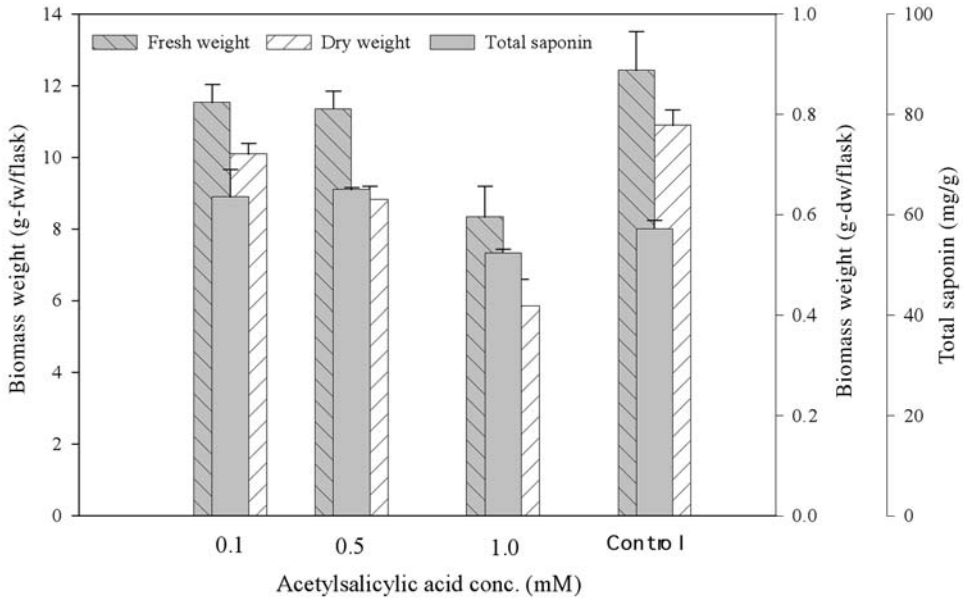

Fig. 4. Effect of acetylsalicylic acid on metabolite accumulation of hairy roots.

with 1 mL addition, total ginseng saponin content increased to as much as 66.9±1.3 mg/g of dry wt compared with 57.2±1.6 mg/g in the control (about 1.17 times the control). However, metabolite productivity was similar to the control with the addition of 0.1 mL yeast elicitor. Additions of yeast elicitor greater than 0.1 mL caused a slight drop in metabolite productivity. With 0.5 mL addition, hairy roots grow more than with 0.1 and less than with 1.0 mL addition, whereas total ginseng saponin content is lower. Therefore, this elicitor can act as a growth enhancer at some concentrations.

Chen *et al.* (*14*) reported that upon elicitation with yeast elicitor in *Salvia miltiorrhiza* hairy root culture, the production of both phenolic acids and tanshinones was enhanced and the contents of two phenolic acids, rosmarinic acid and lithospermic acid B, were elevated from 1.24% and 2.59% to 2.89% and 2.98% of dry wt, respectively, while the intracellular content of cryptotanshinone increased from 0.001% to as much as 0.096% of dry wt. Also, yeast elicitor also improved the growth of hairy roots (from 3.9 g/L to 7.3 g/L on a dry weight basis).

Effect of Bacterial Elicitor on Growth and Secondary Metabolite Production

Because the biosynthesis of secondary metabolites in plants is tightly controlled during development and the metabolites are accumulated by

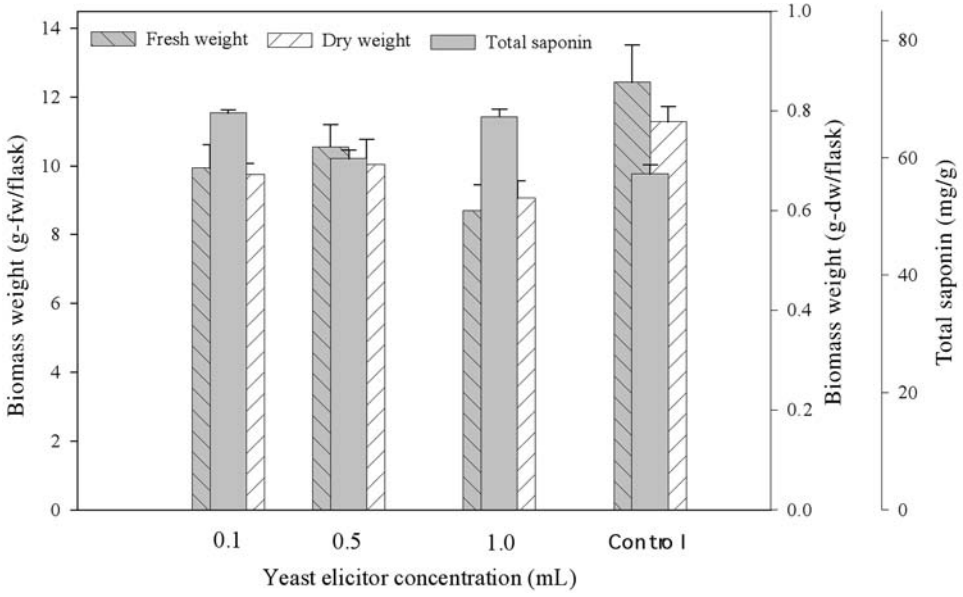

Fig. 5. Effect of yeast elicitor on metabolite accumulation of hairy roots.

plants in response to stress and microbial attack, bacterial elicitor is a good material to study defense response in vitro cultures (*15*).

After 3 d of elicitation, the addition of raw bacterial elicitor was detrimental to the growth of hairy roots as shown in Fig. 6. The use of bacterial elicitor at all three concentrations resulted in a slight decrease in biomass growth, whereas total ginseng saponin content increased with added concentration. In the case of 1 mL addition, total ginseng saponin content increased to 1.23 times the control (57.2±1.6 mg/g to as much as 70.7±2.6 mg/g in the control). However, metabolite productivity was enhanced by about 1.2 times with the 1 mL addition of bacterial elicitor. Raw bacterial elicitor increased total ginseng saponin content and enhanced productivity, while the autoclaved bacterial elicitor did not alter the content of total ginseng saponin. Also, Jung et al. (*15*) reported that raw bacterial elicitors affected the tropane alkaloid profile by increasing the scopolamine concentration, while the autoclaved bacterial elicitors produced similar effects on the control in *Scopolia parviflora* hairy root cultures.

The treatment of bacterial elicitor has some economic and operating advantages compared with other treatment such as yeast elicitor; these advantages are low preparation cost and no requirement for a preparation process. The preparation of bacterial elicitor is easy as the elicitor is applied in conditions of raw or autoclaved whole broth, while that of yeast elicitor requires some processing steps such as repeated ethanol precipitation and

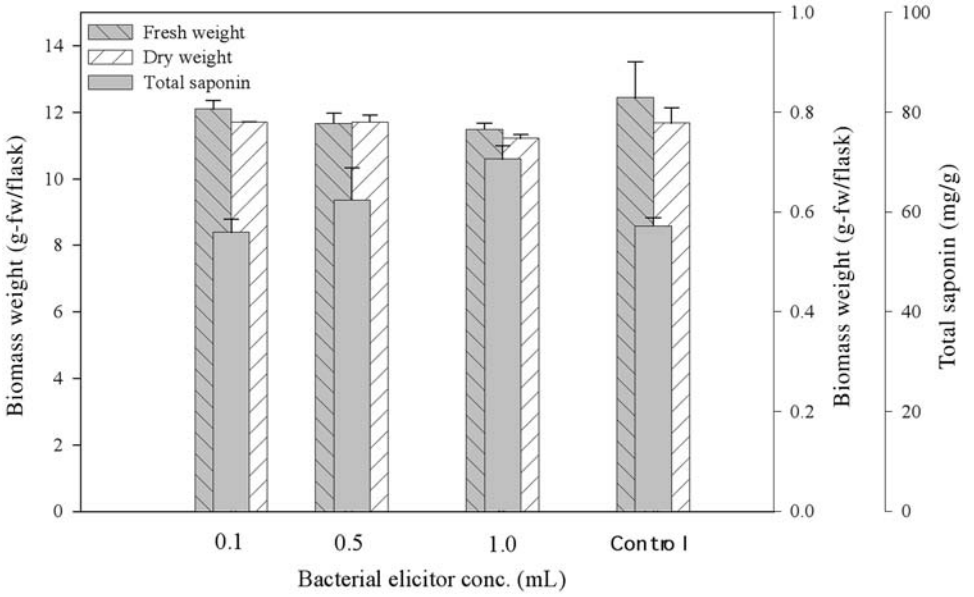

Fig. 6. Effect of bacterial elicitor on metabolite accumulation of hairy roots.

purification steps. Moreover, the preparing time of bacterial elicitor is shorter than that of yeast elicitor; the bacteria were grown within 2 d while the preparation of yeast elicitor took about 7 d. Therefore, the application of bacterial elicitor will make a great contribution to diminution of the production cost in large-scale production of ginseng saponin (ginsenosides).

Conclusions

Plant hairy root culture has been shown to be feasible for *in vitro* production of secondary metabolites. *P. ginseng* C.A. Meyer is a one of the most famous oriental medicinal plants. They have many beneficial bioactive compounds. Hairy root cultures have several advantages over field cultures. In this article, we investigated the effect of several elicitors on the growth and metabolite biosynthesis of *P. ginseng* C.A. Meyer hairy root culture. Every elicitation experiment (SA, ASA, yeast elicitor, and bacterial elicitor) inhibited the growth of hairy roots, whereas ginseng saponin content was enhanced by the treatments. These results indicate that elicitation strategy can reduce the processing time needed to achieve high levels of useful metabolites in hairy root culture.

Acknowledgment

This study was financially supported by Special Research Fund of Chonnam National University in 2004.

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