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Enhancement of Growth and Secondary Metabolite Biosynthesis: Effect of Elicitors Derived from Plants and Insects

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Abstract Plant-derived natural products have been and will continue to be valuable sources. Elicitors have been employed to modify cell metabolism in order to enhance the productivity of useful metabolites in plant cell/tissue cultures. In this study, several elicitors were used to improve the productivity of useful metabolites and to reduce culture time for archiving high concentration in *P. ginseng* hairy root cultures. The addition of chitosan, chitosan oligosaccharide and alginate oligosaccharide to the culture of *P. ginseng* hairy roots caused growth to be inhibited with the increase in elicitor concentration. The usage of the chitosan elicitor and D-glucosamine caused a slight decrease in hairy root growth, whereas total ginseng saponin accumulated slightly with the increase in elicitor concentration. When gel beads were added to the culture medium at the initial period, hairy root growth was enhanced. The maximum growth was 1.35 times higher than that of the control at 1% (w/v). Total ginseng saponin content decreased due to the addition of alginate beads. This would result in consistent diffusion of lower levels of calcium ions during the culture period that promotes biomass growth.

Keywords: *Panax ginseng*, hairy root, elicitor, chitosan, alginate bead, secondary metabolite

INTRODUCTION

Plants are a potential source of a large number of valuable constituents [1-3]. Generally, plants produce secondary metabolites in nature as a defense mechanism against attack by pathogens and insects. In recent research of the root culture system, a wide variety of elicitors have been employed to modify cell metabolism in order to enhance the productivity of useful metabolites in plant cell/tissue cultures. The cultivation period in particular, may be reduced to achieve high product concentrations [4,5].

Elicitors promote the formation of secondary metabolites. Elicitation strategies are compounds or treatments that induce plants to synthesize phytoalexins at elevated levels [4]. The active mechanisms of elicitors are considered to be different and complex. Since little is known of the biosynthetic pathways of most secondary metabolites in plants, the effect of elicitation on a plant cell/tissue culture cannot be easily predicted. Therefore, elicitation approaches are performed by trial and error. The effect of elicitors depends on many factors, such as the concentration of the elicitor, the growth stage of the culture at the time of elicitation and the contact time of elicitation [6].

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Endogenous elicitors originating from plant structural compounds, oligouronic acids, oligogalacturonic acid and lignin-like compounds, have been separated and purified as elicitors. However, it is not yet known how it acts in plant cells [7]. The biological effects elicited in plants by oligosaccharides are diverse. Oligosaccharides induce responses including stem elongation, stimulation of ethylene production, antagonizing auxin and the elicitation of various defensive actions. The structurally defined fragments of plant cell wall polysaccharides are able to function in plant cell/tissue cultures as chemical messages with specific regulatory properties [8].

Exogenous elicitors are derived from microorganisms or insects. Chitosan and its oligomer (components of mycelium cell wall), β-glucan and glycoprotein show strong elicitor activities [7]. Chitosan (β-1,4-linked glucosamine) and its oligomer proved to be very effective elicitors in several plant suspension cultures [7,9].

Hairy roots induced by *Agrobacterium rhizogenes* are a valuable and promising source of root-derived phytochemicals that are useful as pharmaceuticals, cosmetics, pigments, and food additives. Transformed hairy roots of many plant species have been widely investigated to improve the *in vitro* biosynthesis of secondary metabolites. In the near future, this approach may be a reality for the commercial production of useful compounds using a transgenic hairy root culture system derived from plants [10,11].

Panax ginseng C.A. Meyer is one of the most famous oriental medicinal plants and has many beneficial bioactive effects on human health. The major compounds of pharmaceutical activation are identified to be saponin (ginsenosides), phenolic compounds and polysaccharides [10].

In this study, *P. ginseng* C.A. Meyer hairy root cultures, established by infecting with *A. rhizogenes* KCTC 2744, were used for improving the biosynthesis of a secondary metabolite by using several elicitors (chitosan, alginate, *etc.*).

MATERIALS AND METHODS

Hairy Roots and Culture

The hairy roots of *P. ginseng* C.A. Meyer were initiated and maintained as described previously [10]. In all experiments, the hairy roots were cultivated in liquid hormone-free 1/2 MS medium 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°C prior to use. Cultures were incubated at 23°C in the dark in a 250-mL Erlenmeyer flask on a rotary shaking incubator (Vision Scientific, Ltd.) operated at 80 rpm.

Experimental Procedure

Effect of Elicitors on Hairy Root Growth

In order to investigate the effect of agarose, alginic acid, agar and sodium alginate on growth and total ginseng saponin content, each compound was added to 0.1% (w/v) at the initial culture period. Alginate oligosaccharide and chitosan oligosaccharide were added from 0 to 0.1% (w/v) and cultured for 24 days. Chitosan was added from 0 to 1,000 mg/L. Every compound was added to the 1/2 MS medium to an appropriate concentration before autoclaving.

Preparation of Chitosan Elicitor and Dosage Response

The chitosan elicitor was purified by a method described elsewhere. Briefly, it was dissolved in 90 mL of 0.1 N acetic acid, and the solution was centrifuged for 30 min at 3,000 rpm. Then, the insoluble fractions were discarded. This procedure was performed twice. After centrifugation, the supernatant was precipitated by an adjustment of its pH to 7.0 with 2 N NaOH. The precipitates were washed extensively with distilled water and then dried. The purified chitosan was dissolved in 0.1 N acetic acid (1 g chitosan/90 mL acetic acid) and the pH of the solution was adjusted to 5.8.

The culture method and medium were the same as were used in previous experiments [10]. After 21 days of cultivation, different concentrations of the purified chitosan elicitor were added to the culture medium. On day 3 of elicitation, hairy roots were harvested and the biomass and metabolite content were determined.

D-glucosamine Dosage Response

The culture method and medium were the same as was used in the chitosan elicitor experiments. After 26 days of cultivation, different concentrations of D-glucosamine were added to the culture medium. On day 3 of elicitation, hairy roots were harvested and the biomass and metabolite content were determined.

Preparation of Gel Beads and Treatment

In making alginate gel beads, 20 mL of 2% (w/v) sodium alginate solution was dropped into 2% (w/v) $CaCl_2$ solution, which was adjusted to pH 5.7. After formation, the gel beads were stabilized by maintaining them in a 1/2 MS medium containing 2% (w/v) $CaCl_2$. The stabilizing solution was removed and the gel beads were washed twice with 1/2 MS medium. Every reagent and solution was autoclaved at 121°C for 15 min before use. Prepared gel beads were composed with diameters of 2~2.5 mm.

For the experiments, the gel beads were added from a 0 to 5% (w/v) concentration into 250-mL flask cultures containing 100 mL of 1/2 MS medium and cultured for 21 days. Hairy roots were harvested and the biomass and metabolite content were determined.

Analytical Methods

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

Extraction and Analysis of Total Ginseng Saponin

To determine total ginseng saponin, 100 mg of powdered dry hairy roots was soaked in 5 mL of *n*-BuOH saturated with distilled water, stored at 4°C for 24 h, sonicated in an ultrasonic cleaning bath for 60 min and centrifuged twice at 10,000 rpm for 10 min. The collected supernatant was used for total ginseng saponin analysis. Total ginseng saponin was measured by the Vanillin-H_2SO_4 colorimetric method [10]. A calibration curve was established with a ginsenoside Re standard. Authentic ginsenoside Re was purchased from Sigma-Aldrich Co., Ltd (St. Louis, USA).

Calculations of Total Ginseng Saponin Content and Productivity

The total ginseng saponin content of *P. ginseng* hairy roots was calculated as:

Total ginseng saponin content (mg/g)
= total ginseng saponin concentration of sample (mg/L) × sample volume (L) / used hairy root dry weight (g)

The total ginseng saponin productivity of *P. ginseng* hairy roots was calculated as:

Total ginseng saponin productivity (mg/L)
= total ginseng saponin content (mg/g) × harvested hairy root dry weight (g) / volume of culture medium (L)

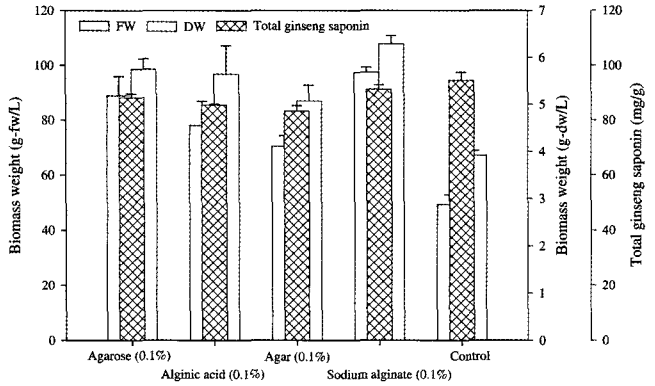

Fig. 1. Effect of agarose, alginic acid, agar and sodium alginate on the growth and total ginseng saponin content of *P. ginseng* hairy roots.

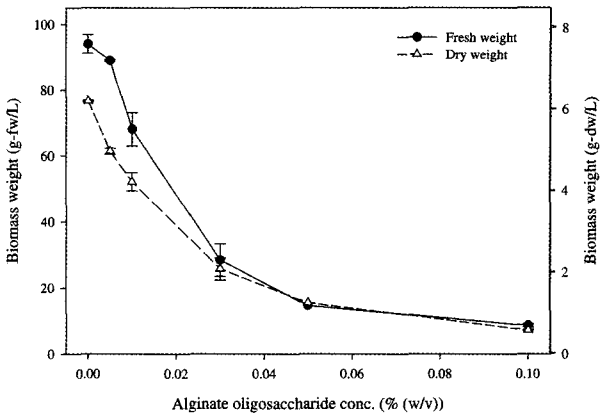

Fig. 2. Effect of alginate oligosaccharide on the growth of *P. ginseng* hairy roots.

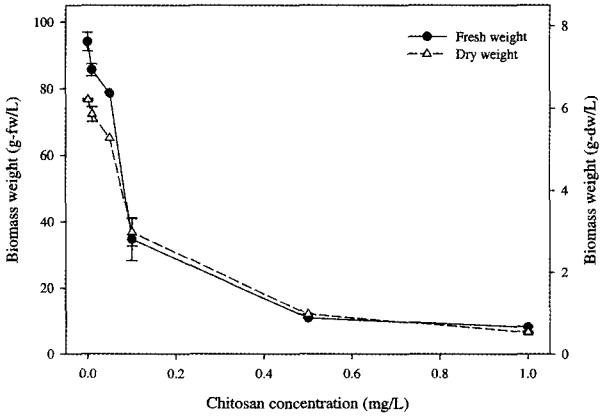

Fig. 3. Effect of chitosan on the growth of *P. ginseng* hairy roots.

RESULTS AND DISCUSSION

Effect of Agarose, Alginic Acid, Agar, Sodium Alginate and Its Dligomers on Growth and Secondary Metabolite Accumulation

Some reports have indicated that alginate, which is not a component of plant cell walls, functions as an elicitor-like substance. However, it is not yet known how it acts in plant cells [7]. The biological effects elicited in plants by oligosaccharides are diverse. Oligosaccharides induce responses including stem elongation, stimulation of ethylene production, antagonizing auxin and the elicitation of various defensive actions. The structurally defined fragments of plant cell wall polysaccharides are able to function in plant cell/tissue cultures as chemical messages with specific regulatory properties [8].

Fig. 1 shows the effect of agarose, alginic acid, agar and Na-alginate on the growth and secondary metabolite accumulation of *P. ginseng* hairy roots in 250-mL flask cultures. The addition of 0.1% (w/v) agarose, alginic acid, agar and Na-alginate enhanced biomass growth in hairy root cultures. Total ginseng saponin contents of each material were slightly lower than that of the control. Sudha and Ravishankar [8] reported that sodium alginate enhances the production of capsaicin in cell cultures of *Capsicum frutescens*.

Fig. 2 shows the effect of alginate oligosaccharide (AO) on the growth of hairy roots in 250-mL flask cultures. When AO was added to the culture medium, hairy root growth was inhibited significantly with an increase in AO concentration. Also, the addition of AO did not result in the enhancement of total ginseng saponin content at the tested concentration (as much as 75.86 ± 5.8~77.61 ± 2.1 mg/g compared with 78.64 ± 1.57 mg/g in the control). But in the case of a 0.005% (w/v) addition, metabolite productivity decreased to about 0.77 times that of the control. However, Akimoto *et al.* [7] reported that the addition of the alginate oligomer to a suspension culture of *Cath. roseus* L. or *Wasabia japonica* cells promotes the production of antibiotic enzymes. They concluded that alginate acts as an endogenous elicitor. Also, Natsume *et al.* [12] reported in a barley bioassay that alginate-derived oligosaccharides have a root growth-promoting activity.

Effects of Chitosan and D-Glucosamine on Growth and Secondary Metabolite Accumulation

Chitosan (β-1,4-linked glucosamine) and its oligomer, which are components of the mycelium cell wall, proved to be very effective elicitors in several plant suspension cultures [7,9]. Fig. 3 shows the effect of chitosan on the growth of hairy roots in 250-mL flask cultures. When chitosan was initially added to the culture medium, hairy root growth was inhibited significantly with the increase in chitosan concentration. However, total ginseng saponin content was enhanced relative to that of the control (data not shown). The addition of 100 mg/L chitosan resulted in a total ginseng saponin content of 86.07 ± 0.74 mg/g (1.1 times that of the control). These results indicate that the addition of chitosan can be used to enhance production of metabolite.

The chitosan elicitor (0.01 to 0.1 g/L) was adminis-

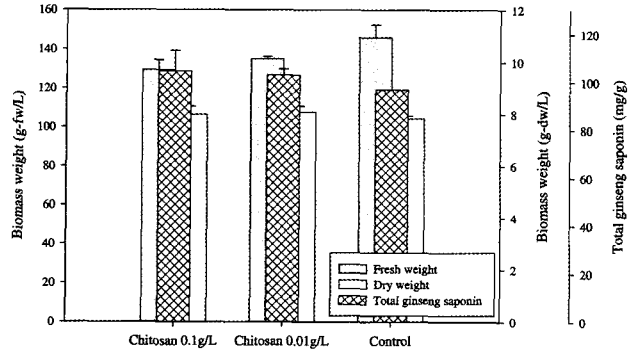

Fig. 4. Effect of the chitosan elicitor on the growth and total ginseng saponin accumulation of *P. ginseng* hairy roots.

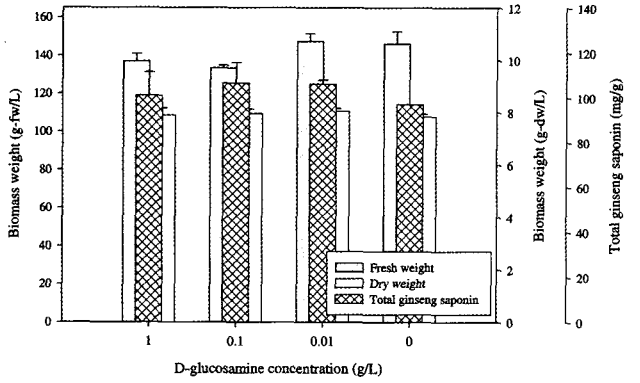

Fig. 5. Effect of D-glucosamine on the growth and total ginseng saponin accumulation of *P. ginseng* hairy roots.

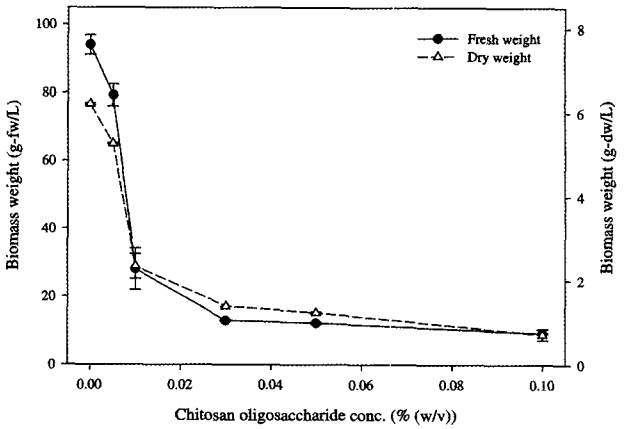

Fig. 6. Effect of chitosan oligosaccharide on the growth of *P. ginseng* hairy roots.

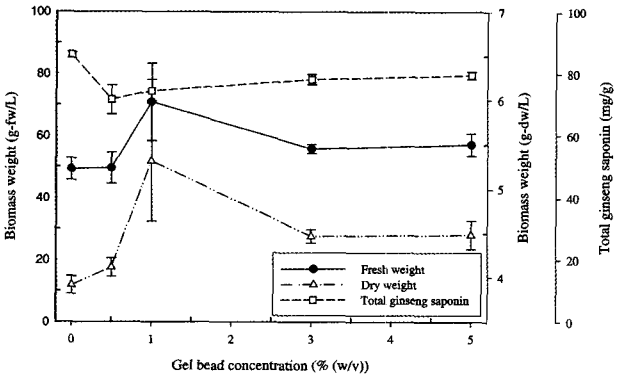

Fig. 7. Effect of gel beads on the growth and total ginseng saponin content of *P. ginseng* hairy roots.

trated after 21 days of the culture hairy root growth was slightly decreased by the chitosan elicitor as shown in Fig. 4. However, total ginseng saponin content increased slightly with the increase in elicitor concentration. The total ginseng saponin productivity was enhanced to about 1.10 times that of the control (as much as 834 mg/L compared with 758.7 mg/L for the control).

In this experiment, D-glucosamine, a monomer of chitosan, was applied as an elicitor in *P. ginseng* hairy root cultures. Fig. 5 shows the effect of D-glucosamine on the growth and total ginseng saponin biosynthesis of hairy roots in 250-mL flask cultures. When D-glucosamine was added to the culture medium, hairy root growth on a fresh weight basis was inhibited slightly with the increase in D-glucosamine concentration. Dry weight was slightly increased by the addition of D-glucosamine. This means that D-glucosamine affects cell membrane activity, and subsequently causes a decrease in water content. Total ginseng saponin content and productivity were enhanced to about 1.10 and 1.12 times that of the control, respectively.

Oligosaccharides of both a fungal and plant origin, derived from β-glucan, xyloglucan, chitin and pectin, have been reported to be potent signaling molecules that regulate growth, development and defense reactions in plants [12]. Fig. 6 shows the effect of chitosan oligosaccharide (CO) on the growth of hairy roots in 250-mL flask cultures. When CO was added to the culture medium initially, hairy root growth was inhibited significantly with the increase in CO concentration. Total ginseng saponin content was similar to that of the control at a 0.005% CO addition. However, productivity was lower than that of the control (0.85 times the productivity of the control).

Effect of Gel Beads on Growth and Secondary Metabolite Production

Some reports showed that alginate acts as an elicitor-substance, but the mechanism by which it affects plant cells has not been revealed [7]. Fig. 7 shows the effect of alginate gel beads on the growth and total ginseng saponin accumulation of hairy roots in 250-mL flask cultures. When gel beads were added to the culture medium at the initial period, hairy root growth was enhanced. The maximum growth of hairy roots was 1.35 times higher than that of the control at 1% (w/v). Total ginseng saponin content decreased due to the addition of alginate beads. Alginate gel beads are saturated with high levels of

calcium before being added to a culture medium. The excess calcium may diffuse outward into the culture medium. This would result in consistent diffusion of lower levels of calcium ions during the culture period that promotes biomass growth.

CONCLUSION

Plant hairy root culture has been shown to be feasible for *in vitro* production of secondary metabolites. In this paper, the effect of several elicitors on the growth and secondary metabolite production of *P. ginseng* hairy root was investigated. Every elicitation experiment inhibited the growth of hairy roots, whereas the ginseng saponin content was enhanced by the treatments. These results indicate that processing time can be reduced to achieve high levels of a useful metabolite by the use of an elicitation strategy and hairy root culture.

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