0273-2289/06/129–132/436–446/$30.00

Enhanced Secondary Metabolite Biosynthesis by Elicitation in Transformed Plant Root System

Effect of Abiotic Elicitors

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Abstract

Plants generally produce secondary metabolites in nature as a defense mechanism against pathogenic and insect attack. In this study, we applied several abiotic elicitors in order to enhance growth and ginseng saponin biosynthesis in the hairy roots of *Panax ginseng*. Generally, elicitor treatments were found to inhibit the growth of the hairy roots, although simultaneously enhancing ginseng saponin biosynthesis. Tannic acid profoundly inhibited the hairy root growth during growth period. Also, ginseng saponin content was not significantly different from that of the control. The addition of selenium at inoculum time did not significantly affect ginseng saponin biosynthesis. However, when 0.5 m*M* selenium was added as an elicitor after 21 d of culture, ginseng saponin content and productivity increased to about 1.31 and 1.33 times control levels, respectively. Also, the addition of 20 μ*M* $NiSO_4$ resulted in an increase in ginseng saponin content and productivity, to about 1.20 and 1.23 times control levels, respectively, and also did not inhibit the growth of the roots. Sodium chloride treatment inhibited hairy root growth, except at a concentration of 0.3% (w/v). Increases in the amounts of synthesized ginseng saponin were observed at all concentrations of added sodium chloride. At 0.1% (w/v) sodium chloride, ginseng saponin content and productivity were increased to approx 1.15 and 1.13 times control values, respectively. These results suggest that processing time for the generation of ginseng saponin in a hairy root culture can be reduced via the application of an elicitor.

Index Entries: Elicitor; selenium; nickel; ginseng saponin; hairy roots.

Introduction

Plants constitute a large source of valuable compounds. About 100,000 compounds have been isolated from plant sources, with about 4000 new

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compounds being discovered every year. These compounds not only are connected with important traits of the plant itself, including color, fragrance, taste, and resistance to pests and diseases, but also may prove useful in the manufacture of a host of useful products, including drugs, antioxidants, flavorings, fragrances, pigments, insecticides, and many other important industrial and medicinal raw materials *(1,2)*. Plant cell and tissue cultures are an attractive alternative to the cultivation of whole plants for production of valuable secondary metabolites *(3)*.

The hairy roots induced by *Rhizobium rhizogenes* constitute a valuable and promising source of root-derived phytochemicals. These transformed hairy roots can synthesize fairly robust amounts of several metabolites, at levels similar to, or even greater than, the amounts that would be generated by the original plants *(4)*. Hairy roots are characterized by high growth rates, high metabolite productivity, and inherent genetic stability *(5,6)*. In the near future, the plant-derived transgenic hairy root-based production approach may become a widely used method, allowing for the commercial production of a myriad of useful compounds *(7)*.

In general, plants produce secondary metabolites in nature as a defense mechanism against pathogenic and insect attack. In recent research into in vitro culture systems, a wide variety of elicitors have been employed in order to modify cell metabolism. These modifications are designed to enhance the productivity of useful metabolites in the cultures of the plant cells/tissues. The cultivation period, in particular, can be reduced by the application of elicitors, although maintaining high concentrations of product *(8,9)*. Elicitation strategies are compounds or treatments which cause plants to synthesize elevated levels of phytoalexins *(8)*. The active mechanisms employed by elicitors are complex and distinctive. As little is known regarding the biosynthetic pathways of most secondary plant metabolites, the effects of elicitation on a plant cell/tissue culture are difficult to predict. Therefore, elicitation approaches tend to be empirical steps. The effects of elicitors rely on a host of factors, including the concentration of the elicitor, the growth stage of the culture at the time of elicitation, and the contact duration of elicitation *(10)*. Both biotic and abiotic elicitors can be used to stimulate secondary metabolite biosynthesis in plant cell/tissue cultures, thereby reducing the processing time necessary for high product yields. Elicitors of nonbiological origin (abiotic elicitors), such as heavy metals and ultraviolet light, which induce phytoalexin synthesis, are generally designated as abiotic stresses *(8,10)*.

In previous researches, tannic acid, selenium, and $NiSO_4$ despite its functionality were not introduced to elicitation process for enhancing of secondary metabolites in plant cell/tissue cultures. Tannic acid, a commercial form of tannin, is not actually a true acid, but rather an acid-like substance, called a polyphenol. Tannic acid occurs naturally in tea, coffee, oak, and sumac bark. Tannin molecules crosslink proteins, rendering the tissues more resistant to both bacterial and fungal attacks. Many tannins are

known to be cytotoxic to cell cultures. Tannins may also exhibit antibiotic activity that appears to be resulting from the precipitation of pathogen-produced extracellular enzymes, or interference with the metabolism of the pathogen *(11)*. For many years, selenium (Se) has been recognized as an essential trace element for both humans and livestock. Selenium has also been recognized to be an important cofactor in several selenoproteins. In order to increase selenium intake using natural sources, selenium content must be increased in cultivated crops. Supplementation is normally accomplished by the addition of either selenite or selenate to fertilizers, the spraying of crops with selenium salts, or treatment of the seeds with aqueous selenium. However, selenium is not an essential nutrient for plants. Also, the effects of selenium on plant growth and metabolite biosynthesis have yet to be thoroughly investigated *(12)*. Nickel (Ni) has been identified as an essential element for plant growth. Ni is required for the activation of functional urease, because it is a cofactor. To the best of our knowledge, the activation of urease is the sole proven function of Ni in higher plants. There have also, however, been some reports regarding the stimulatory effects of Ni on plant growth *(13,14)*. Salinity may affect a variety of metabolic processes, including photosynthesis, protein synthesis, respiration, nitrogen assimilation, and the turnover of plant growth regulators. Cytokinin synthesis in the roots and cytokinin transport to the shoots is also influenced by salinity, but abscisic acid synthesis is promoted *(15)*.

Panax ginseng C. A. Meyer, a member of the Araliaceae family, is one of the better known oriental medicinal plants and is widely distributed throughout the Korean peninsula and China. Ginseng plants have been reported to exert a variety of beneficial bioactive effects on human health including hemostatic qualities. Ginseng plants also appear to exhibit properties that promote blood circulation, relieve pain, stanch bleeding wounds and alleviate trauma, relieve stress, and improve immune function. A great deal of chemical, biochemical, and pharmacological research into the properties of ginseng plants has been recently carried out *(16,17)*. The principal compounds involved in the pharmaceutical effects of ginseng have been isolated and identified in a host of previous studies. These compounds include ginseng saponin (ginsenosides), polysaccharides, antioxidants, peptides, fatty acids, alcohols, vitamins, and phenolic compounds. The pharmacological characteristics and effects of the ginsenosides Rb_1 and Rg_1 are distinct, and are sometimes antagonistic. Ginsenoside Rb_1 manifests sedative, anticonvulsive, analgesic, antipyretic, anti-inflammatory, and antipsychotic properties, and also has been shown to improve gastrointestinal motility. Ginsenoside Rg_1 exerts stimulant and antifatigue effects, and also appears to enhance motor ability. In recent years, ginseng polysaccharides have been identified as potentially useful compounds that appear to exert important pharmacological effects. Ginseng polysaccharides also apparently possess immune stimulation, antitumor, antihepatitis, and mitogenic and hypoglycemic properties *(5,16,17)*.

It is known to that the ginseng saponin of *P. ginseng* was generated at the latter culture period. This biosynthesis pattern, nongrowth associated process, is well known regarding the formation of secondary metabolites in plant cell/tissue cultures. In this study, *P. ginseng* C.A. Meyer hairy root cultures, generated by infection with *R. rhizogenes* KCTC 2744, were employed in order to enhance the biosynthesis of a secondary metabolite, via the application of abiotic elicitors.

Materials and Methods

Hairy Roots and Culture

The hairy roots of *P. ginseng* C. A. Meyer were induced and maintained, as previously described *(18)*. In all of the experiments in this study, the hairy roots were cultivated in liquid hormone-free 1/2 MS medium that contained 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 before use. The cultures were then incubated at 23 ± 1°C in darkness in a 250 mL Erlenmeyer flask, on a rotary shaking incubator at 80 rpm.

Experimental Procedures

In order to determine the effects of several elicitors on hairy root growth and ginseng saponin biosynthesis in *P. ginseng* hairy roots in 250 mL flask cultures, tannic acid (0–10 m*M*), selenium (as selenite; 0–10 mg/L), nickel (as $NiSO_4$; 0–50 μ*M*), NaCl (0–0.3% [w/v]), and some other abiotic elicitors (ascorbic acid 100 mg/L; salicylic acid [SA] 400 μ*M*; H_2O_2 0.012% [v/v]; $CuSO_4$ 2 m*M*; $CaCl_2$ 20 m*M*; SA 400 μ*M* + $CaCl_2$ 20 m*M*; SA 400 μ*M* + H_2O_2 0.012% [v/v]) were applied. 0.2 mL of elicitors was added to each sample flasks at inoculum time. The pH of the prepared elicitor solutions was adjusted to 5.8, using either 1 *N* NaOH or 1 *N* HCl. At each experiment, the control was incubated to same culture condition for same period.

For selenium elicitation experiments, after 21 d of culture which did not added any elicitor (first culture), different concentrations of the prepared selenium elicitors (as selenite; 0–0.5 m*M*) were added to culture media. On day 3 of elicitation (second culture), the hairy roots were harvested, and the biomass and ginseng saponin content were measured. To compare with the elicitation effect of elicitor, the control was incubated to same culture condition for 24 d. All experiments are performed at least two independent experiments.

Analytical Methods

In order to determine the weight of the biomass, the hairy roots were harvested and rinsed with distilled water, and blotted to remove extra water and weighed. Dry weight was measured gravimetrically after drying the roots for 24 h at 60°C. Results are expressed as the mean value of at least two independent measurements.

Extraction and Analysis of Ginseng Saponin

In order to quantify the total amount of biosynthesized ginseng saponin, 100 mg of powdered dry hairy roots were soaked in 5 mL of distilled water-saturated *n*-butanol stored at 4°C for 24 h, sonicated in an ultrasonic cleaning bath for 1 h, and centrifuged twice at 5030*g* for 10 min. The collected supernatants were then used to determine the total amount of ginseng saponin that had been synthesized. Ginseng saponin content was measured via Vanillin-H_2SO_4 colorimetry *(19)*. A calibration curve was established using a ginsenoside Re standard. Authentic ginsenoside Re was purchased from Sigma-Aldrich Co., Ltd (St. Louis, MO).

Calculations of Ginseng Saponin Content and Productivity

The ginseng saponin content of the *P. ginseng* hairy roots was calculated as according to the following formula:

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

The ginseng saponin productivity of the *P. ginseng* hairy roots was calculated according to the following formula:

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

Results and Discussion

Effect of Tannic Acid, Selenium, $NiSO_4$, and NaCl on Growth and Secondary Metabolite Production

Growth and ginseng saponin formation of *P. ginseng* hairy roots was monitored after tannic acid addition to cultures at inoculum time. Tannic acid was found to exert a potent inhibitory effect on hairy root growth (Fig. 1). When tannic acid was added in excess of 0.1 m*M*, serious inhibition of the growth of the hairy roots resulted. The addition of 0.01 m*M* tannic acid resulted in a ginseng saponin content (60.2 ± 4.3 mg/g) not significantly different from that of the control (60.3 ± 1.7 mg/g).

In order to investigate the effect of selenium on *P. ginseng* biomass and ginseng saponin biosynthesis, prepared selenium was added to medium at inoculum time. Selenium was found to strongly inhibit hairy root growth, but caused a slight increase in ginseng saponin content, as is shown in Fig. 2. The addition of 0.1 m*M* selenium resulted in a ginseng saponin content of 80.5 ± 4.4 mg/g (1.07 times control level), and a productivity of 402.3 mg/L (1.04 times control level). These results indicate that the addition of selenium at inoculum time do not greatly improve the productivity of ginseng saponin in *P. ginseng* hairy root cultures. Carvalho et al. *(12)* reported similar results that the effects of selenium supplementation

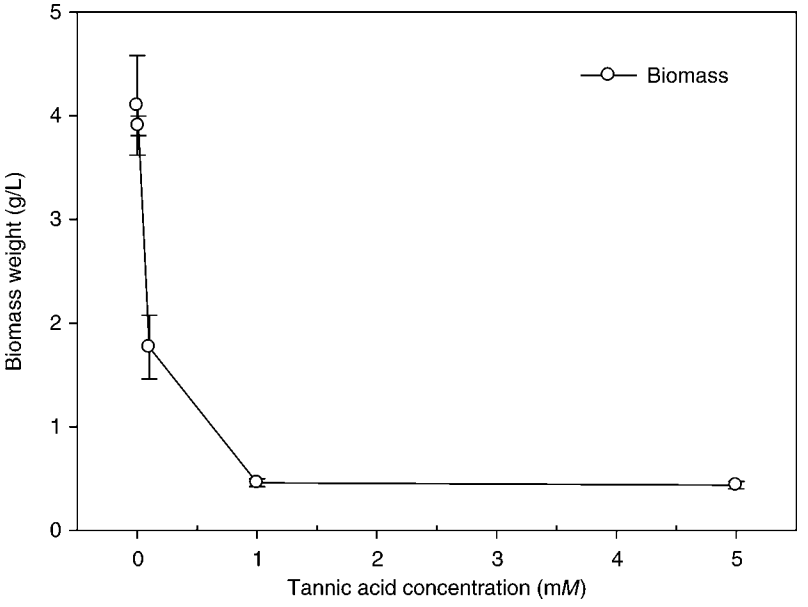

Fig. 1. Effect of tannic acid on growth of hairy roots.

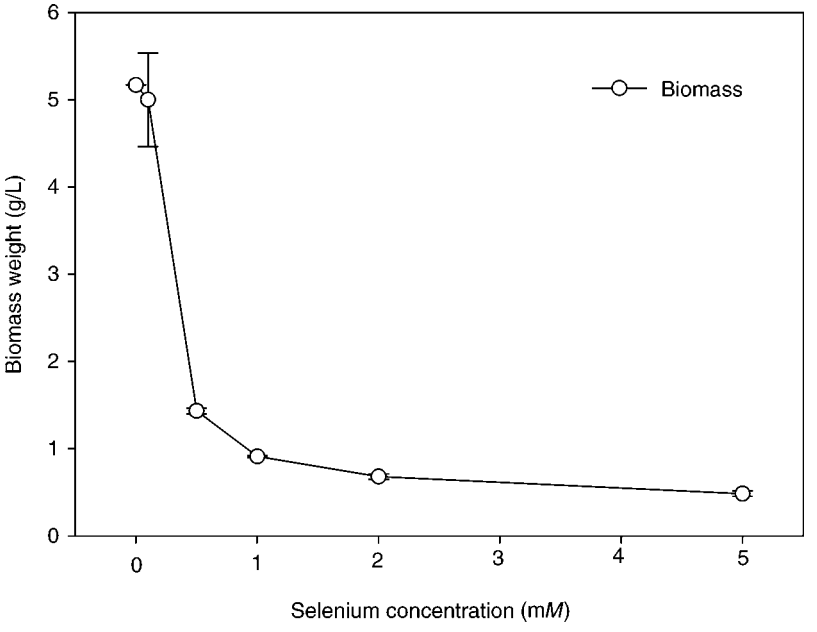

Fig. 2. Effect of selenium on growth of hairy roots.

on germination and plant growth and at sufficiently high concentrations, it can inhibit both the growth and germination of seeds.

As shown in Fig. 3, elicitation via the addition of 0.1 m*M* Se resulted in inhibited biomass growth. However, when 0.5 m*M* Se was added, biomass

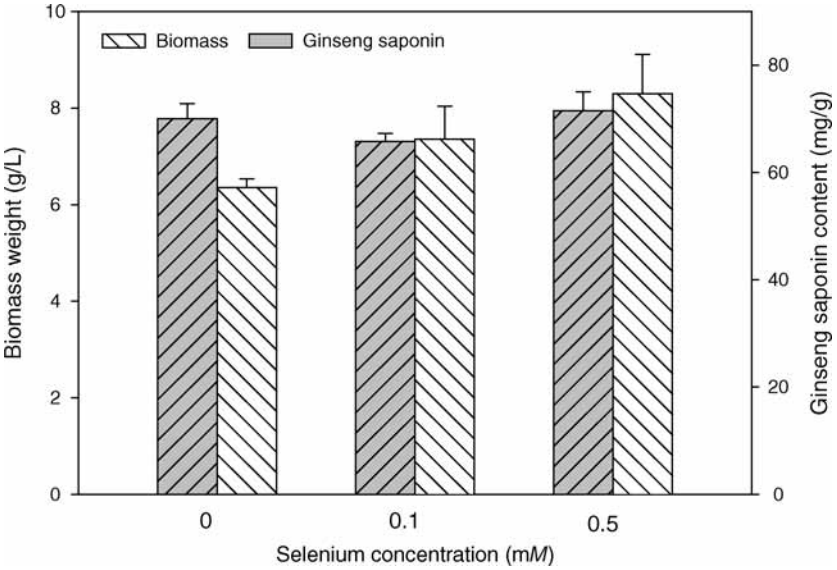

Fig. 3. Effect of selenium elicitor on growth and secondary metabolite accumulation of hairy roots.

was slightly higher than that observed in the control sample. The ginseng saponin content of hairy roots increased directly with increases in the added amount. As a result of the addition of 0.5 m*M* Se, ginseng saponin content was 74.7 ± 7.3 mg/g (1.31 times control value), and the productivity was 594 mg/L (1.33 times control value). Treatment with selenium as an elicitor is associated with several advantages in terms of both economy and operation compared with other treatments such as yeast elicitor treatments. Chief among these advantages are the low preparation cost and the ease of the preparation process. Preparation of a selenium elicitor is fairly simple, because the elicitor is applied as a solution, whereas the preparation of a yeast elicitor requires several processing steps, including repeated ethanol precipitation and purification steps. Also, the time required for preparation of a selenium elicitor is much less than that required for the preparation of yeast elicitor; the preparation of a yeast elicitor takes approx 1 wk. Therefore, application of selenium as an elicitor may dramatically reduce the costs associated with the large-scale production of ginseng saponin (ginsenosides).

In order to determine the influence of $NiSO_4$ on *P. ginseng* hairy root growth and ginseng saponin biosynthesis, different concentrations of $NiSO_4$ were introduced to the medium at inoculum time. Biomass growth, based on the dry weight of the hairy roots, was found to occur properly with the addition of 10 μ*M* $NiSO_4$ as is shown in Fig. 4. Across the entire range of concentration, we observed no serious inhibition of hairy root growth. At less than 10 m*M*, the ginseng saponin content fluctuated. The addition of 20 μ*M* $NiSO_4$ resulted in a ginseng saponin content of 91.6 ± 9.8 mg/g (1.20 times control value) and a productivity of 597 mg/L (1.23 times control value), with no inhibitory effects on hairy root growth.

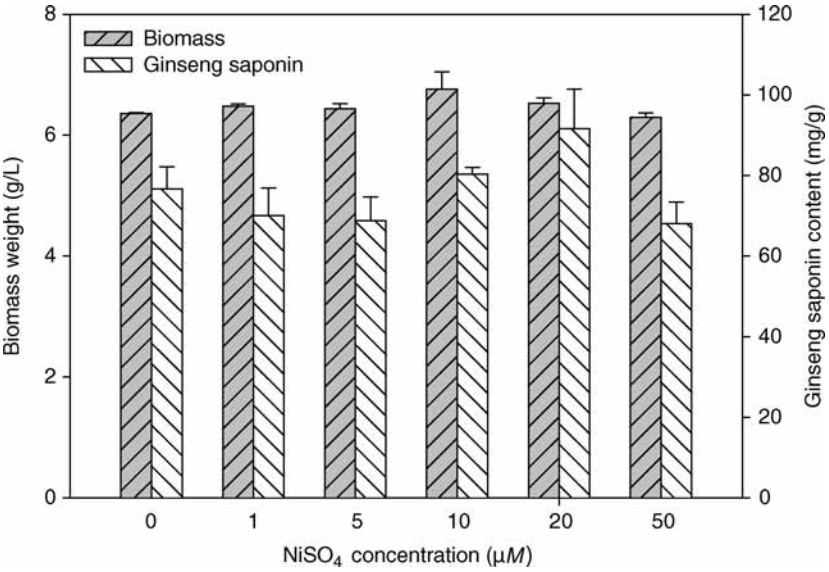

Fig. 4. Effect of $NiSO_4$ on growth and secondary metabolite production of hairy roots.

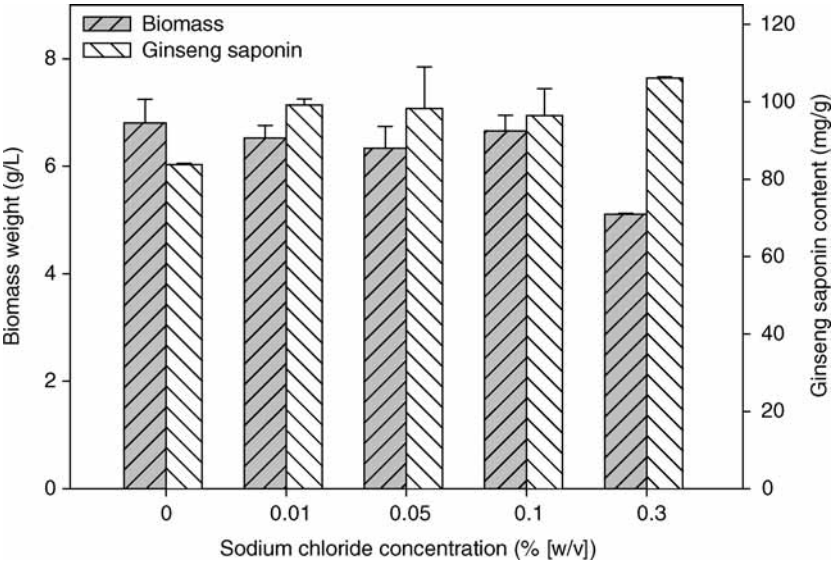

Fig. 5. Effect of sodium chloride on growth and secondary metabolite production of hairy roots.

In order to characterize the effects of sodium chloride on growth and ginseng saponin production in the *P. ginseng* hairy roots, 0–0.3% (w/v) sodium chloride was added to the medium at inoculum time. As shown in Fig. 5, sodium chloride was found to inhibit the hairy root growth, at a concentration of 0.3% (w/v). The addition of sodium chloride less than

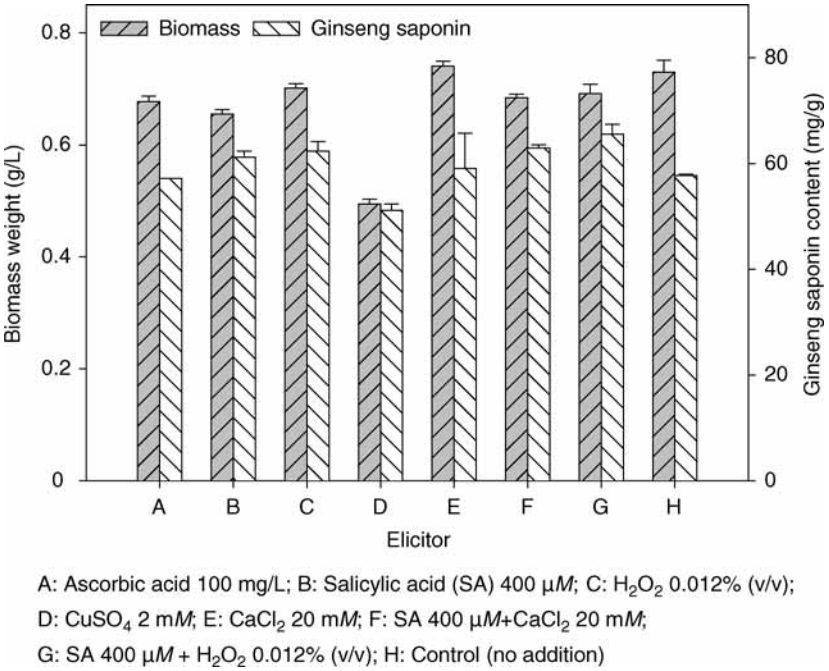

Fig. 6. Effect of several elicitors on growth and secondary metabolite accumulation of hairy roots.

0.1% (w/v) enhanced ginseng saponin content. The addition of 0.1% (w/v) sodium chloride resulted in a ginseng saponin content of 96.4 ± 6.9 mg/g (1.15 times control value) and a productivity of 642 mg/L (1.13 times control value), exerting no observable inhibitory effects on the growth of the hairy roots.

Effects of Some Other Abiotic Elicitors on Growth and Secondary Metabolite Production

As shown in Fig. 6, elicitation was performed with several abiotic compounds added to medium at inoculum time in flask cultures. Ascorbic acid had no enhancing effect on biomass accumulation, but did not inhibit accumulation of ginseng saponin. Other treatments, including 400 μ*M* SA, 0.012% (v/v) H_2O_2, 400 μ*M* SA + 20 m*M* $CaCl_2$, and 400 μ*M* SA + 0.012% (v/v) H_2O_2 treatments resulted in slight increases in ginseng saponin accumulation, as compared with control values. Elicitation with 20 m*M* $CaCl_2$ resulted in slight enhancing in biomass growth and ginseng saponin formation. As a result, salicylic acid, hydrogen peroxide, and calcium chloride can apply to enhancement of secondary metabolite formation in plant hairy root cultures. In the case of 2 m*M* $CuSO_4$ addition, biomass and ginseng saponin accumulation was declined.

Conclusions

Plant hairy root culture appears to constitute a feasible technique for the in vitro production of valuable secondary metabolites. In this article, we attempted to determine the effects of several elicitors on growth and ginseng saponin biosynthesis of *P. ginseng* hairy root cultures. Some of the attempted elicitor treatments inhibited the growth of hairy roots, but increased ginseng saponin levels. The application of tannic acid profoundly inhibited the hairy root growth during growth period. The addition of selenium during the first culture period did not significantly improve ginseng saponin productivitys. However, at second culture period, on the addition of 0.5 m*M* Se as an elicitor, ginseng saponin content and productivity were increased to 1.31 and 1.33 times the control values, respectively. Throughout the entire range of added $NiSO_4$ concentrations, no profound inhibitory effects on hairy root growth resulted. The addition of 20 μ*M* $NiSO_4$ resulted in a maximum ginseng saponin content and productivity of 1.20 and 1.23 times control values, respectively, but did not inhibit the growth of the cultures. Sodium chloride did not inhibite hairy root growth, except at a concentration of 0.3% (w/v). Ginseng saponin content increased as the result of the addition of sodium chloride, and these increases were observed at all concentrations. The addition of 0.1% (w/v) sodium chloride resulted in a ginseng saponin content and productivity of 1.15 and 1.13 times control values, respectively, but did not inhibit the growth of the hairy roots. The results of our study indicate that cultivation period can be reduced by the use of an elicitor and hairy root culture, making possible the large-scale production of useful metabolites in a much shorter time, and at a lower cost, than is currently possible with cultivation-based methods. However, it is suggested that subsequent studies be conducted to elucidate the enhancement of secondary metabolite productivity.

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