

Methyl Jasmonate Elicitation Enhances Glycyrrhizin Production in *Glycyrrhiza inflata* Hairy Roots Cultures

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Hairy roots were induced by infecting stems and leaves of *Glycyrrhiza inflata* with *Agrobacterium rhizogenes* ATCC 15834. The optimization of growth and glycyrrhizin accumulation of *G. inflata* hairy roots was studied. Sucrose (6%, w/v) was optimal for growth and glycyrrhizin accumulation in *G. inflata* hairy roots. Effects of elicitors like chitosan, methyl jasmonate, and yeast extract on glycyrrhizin production were studied. Methyl jasmonate (100 μ M) was most efficient in enhancing glycyrrhizin production up to almost 109 μ g/g dry weight on day 5 of elicitation. These results indicate that application of elicitors can enhance the capacity of *G. inflata* hairy roots to produce glycyrrhizin.

Key words: Methyl Jasmonate, Glycyrrhizin, *Glycyrrhiza inflata*, Hairy Roots Cultures

Introduction

Glycyrrhiza inflata Batal (Leguminosae) has been used as a source of licorice. Licorice is widely applied in pharmaceutical and food industry (Tomoda *et al.*, 1990). The root of this species contains glycyrrhizin and flavonoids (Haraguchi *et al.*, 1998). Licorice has had a high market demand due to its high medicinal value, whereas the licorice resources in the world regions are limited (Rauchensteiner *et al.*, 2005). Plant *in vitro* culture is an alternative source for the production of valuable secondary metabolites. Hairy roots cultures are valuable sources of medicinal compounds. The interest in hairy roots is due to their ability to grow fast without an external supply of any plant growth regulator. Many studies reported *in vitro* culture of licorice including callus, suspension, hairy roots, and whole plant cultures (Arias-Castro *et al.*, 1993; Ayabe *et al.*, 1990; Henry and Marty, 1984; Hayashi *et al.*, 1988; Li *et al.*, 2000; Shabani *et al.*, 2009; Toivonen and Rosenqvist, 1995; Wang *et al.*, 2010; Wongwicha *et al.*, 2008; Yang *et al.*, 2007). However, there are only a few studies on the production of glycyrrhizin from *in vitro* cultures of licorice. Previously, we induced callus of licorice and successfully detected glycyrrhizin in the callus by the competitive enzyme-

linked immunosorbent assay (ELISA) using anti-glycyrrhizin monoclonal antibody (Wongwicha *et al.*, 2008). As an extension of this approach, hairy roots of *G. inflata* were established and glycyrrhizin production was determined.

It is known that sucrose can affect the growth rate and secondary metabolite production in plant tissue cultures (Dicosmo and Misawa, 1995). Therefore, optimal conditions for hairy roots cultures must be established for the production of biomass and secondary metabolites. Furthermore, biotic and abiotic elicitors are used to stimulate secondary metabolite production in various plant cultures. Elicitation by methyl jasmonate and salicylic acid has increased the production of glycyrrhizin in whole plant cultures of *G. glabra* (Shabani *et al.*, 2009). The effectiveness of elicitation depends on a complex interaction between the elicitor and the plant cell and on elicitor specificity, cell line, and environmental conditions (Smetanska, 2008). In addition, the concentration of elicitor is a factor that strongly affects the intensity of the response, which varies according to the plant species. To enhance the capacity of *G. inflata* hairy roots for glycyrrhizin production, elicitor treatment was investigated to obtain appropriate elicitation conditions that allow the

production of a higher content of glycyrrhizin. In the present study, we report the conditions for optimizing cell growth and glycyrrhizin production and the influence of elicitors on glycyrrhizin accumulation in hairy roots cultures of *G. inflata*.

Material and Methods

Chemicals

Glycyrrhizin was purchased from Wako Pure Chemical (Osaka, Japan). Chitosan was obtained from Fluka Chemical (Buchs, Switzerland). Methyl jasmonate and 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were obtained from Wako Pure Chemical. Salicylic acid was obtained from Sigma (St. Louis, MO, USA). Peroxidase-labeled anti-rabbit IgG was purchased from MP Biomedicals (Solon, OH, USA). Glycyrrhizin-HSA and anti-glycyrrhizin MAb were obtained from Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka, Japan. All other chemicals were standard commercial products of analytical grade.

Plant materials

G. inflata seeds were obtained from the Institute of Botany, Mongolian Academy of Sciences, Ulaanbaatar, Mongolia. The seeds were washed with sterile distilled water and surface-sterilized in 10% sodium hypochlorite for 15–20 min. After being washed three times with sterile water, the seeds were immersed in 70% ethanol for 1 min and then germinated on hormone-free Murashige and Skoog (MS) medium containing 3% sucrose (w/v) at pH 5.5. Germination started within 5 d and plantlets were used for hairy roots induction.

Hairy roots induction

Young stems and leaves of *G. inflata in vitro* plantlets were infected with *Agrobacterium rhizogenes* ATCC 15834. Before infection, the bacteria were cultured in yeast extract broth (YEB) medium at 25 °C for 48 h. The 2- to 3-cm long explants of stems and leaves were infected with *A. rhizogenes* and cultured on MS medium without any antibiotic at 25 °C for 48 h, then transferred to half-strength ($\frac{1}{2}$) MS medium with 500 mg/l cefotaxime (CF). At 2-week-intervals, infected segments were transferred to $\frac{1}{2}$ MS medium with 300 mg/l CF and 100 mg/l CF, respectively. Transformed roots were grown in flasks containing

30 ml of $\frac{1}{2}$ MS medium. The hairy roots were subcultured every 2 weeks into fresh medium.

Growth rates and effects of sucrose on glycyrrhizin accumulation in hairy roots

For study of the growth rate, fully grown hairy roots (0.5 g) were subcultured into 125-ml flasks containing 30 ml of $\frac{1}{2}$ MS liquid medium supplemented with 3% (w/v) sucrose. The medium was agitated on a rotary shaker (100 rpm) and maintained at (25 ± 1) °C under a photoperiod of 16 h with a light intensity of $80 \mu\text{mol}/(\text{m}^2 \text{ s})$ white fluorescent light. Hairy roots were harvested, and their dry weight (wt) and glycyrrhizin content were determined every week. Various contents of sucrose (0–12%, w/v) were used to examine the effect on hairy root growth and glycyrrhizin production. After the fourth week of culture, dry weight and glycyrrhizin content of the hairy roots were determined. Each experiment was done in triplicate.

Elicitor preparation

Elicitation was carried out with chitosan (Chi), methyl jasmonate (MJ) or yeast extract (Y). The chitosan solution (10 mg/ml) was prepared by adding glacial acetic acid drop-wise to chitosan at 60 °C within 15 min (final content 2%, v/v). Then the mixture was diluted with de-ionized water, adjusted to pH 5.5, and sterilized by autoclaving. A methyl jasmonate (10 mM) stock solution was prepared in 40% (v/v) ethanol and then filter-sterilized. The yeast extract was dissolved in de-ionized water to afford a stock solution of 100 mg/ml which was autoclaved.

Elicitor treatment

Hairy roots were subcultured into an 125-ml flask containing 30 ml of $\frac{1}{2}$ MS liquid medium, pH 5.5, and grown using a light intensity of $80 \mu\text{mol}/(\text{m}^2 \text{ s})$ for 16 h/d at 25 °C under agitation (100 rpm) to study the effect of the elicitors. After 14 d of culture, various concentrations of elicitors, *i.e.* methyl jasmonate (50, 100 or 200 μM), yeast extract (0.5, 1 or 2 mg/ml) or chitosan (50, 100 or 150 mg/l), were added to the cultures. Then, hairy roots were harvested after 1, 3, 5 or 7 d of elicitor treatment. All treatments were performed in triplicate.

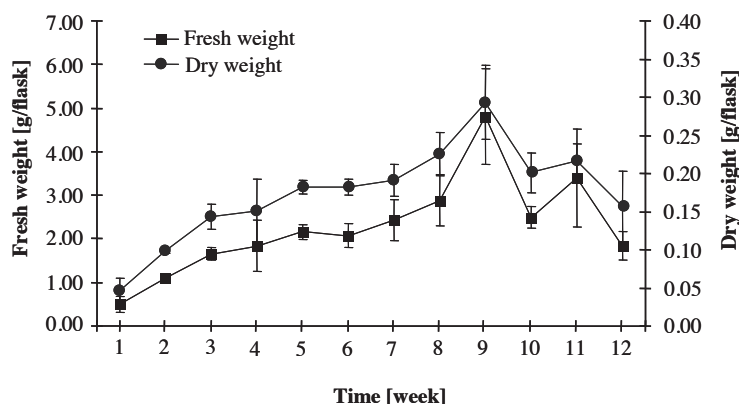


Fig. 1. Time course of the growth of hairy roots originated from *G. inflata* cultured in liquid $\frac{1}{2}$ MS medium for 12 weeks.

Sample preparation and glycyrrhizin analysis

Hairy roots were dried in a hot air oven at 50 °C for 48 h and ground to powder. Dried powder samples (50 mg) were extracted five times with 0.5 ml methanol using an ultrasonic bath for 15 min each time. The extracts were combined and evaporated at 60 °C. The residual solid was dissolved in 1 ml methanol. The extracted solutions were diluted with 20% methanol for determination of glycyrrhizin by indirect competitive ELISA using anti-glycyrrhizin monoclonal antibody as described previously (Shan *et al.*, 2001).

Statistical analysis

Results are reported as the mean \pm standard deviation (SD). The data were analysed statistically by analysis of variance (ANOVA), and the difference between the means of samples was analysed by the least significant difference (LSD) at $P < 0.01$ level.

Results and Discussion

G. inflata *in vitro* plantlets were infected with *A. rhizogenes* ATCC 15834. Transformed roots of *G. inflata* were grown in $\frac{1}{2}$ MS liquid medium without hormones. The hairy roots grew vigorously in the medium and had characteristics of transformed roots such as fast growth and high lateral branching. As shown in Fig. 1, the growth pattern of *G. inflata* hairy roots revealed that the hairy roots grew slowly in the first week of culture. However, they grew faster during days 7–21. The maximum biomass of the hairy root culture was attained

after nine weeks of culture [(0.29 \pm 0.05) g/flask dry wt]. After eight weeks of culture, the colour of the hairy roots changed from light yellow to brown while their fresh and dry weight decreased after nine weeks. The pattern of glycyrrhizin production was the same as that of the growth rate. The glycyrrhizin production gradually increased until it reached the maximum level in the fourth week of culture [(34.79 \pm 4.11) μ g/g dry wt], and thereafter the production rate slightly decreased (Fig. 2). From these results, addition of elicitors at day 14 of hairy roots culture was chosen for elicitation because this corresponded to the exponential phase of hairy roots growth.

Effects of sucrose on biomass and glycyrrhizin accumulation in hairy roots were studied. Fig. 3A shows that the biomass increased with an increase

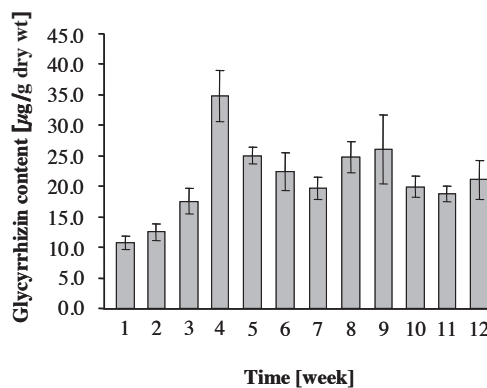


Fig. 2. Time course of the glycyrrhizin content in hairy roots originated from *G. inflata* cultured in liquid $\frac{1}{2}$ MS medium for 12 weeks.

in sucrose content, and the maximum biomass was achieved when 12% sucrose [(0.42 ± 0.02) g/flask] was applied. Glycyrrhizin accumulation was different from biomass accumulation. Without sucrose, hairy roots produced a low yield of glycyrrhizin [(24.68 ± 3.87) $\mu\text{g/g}$ dry wt]. These results confirm that the production of secondary metabolites is affected by the carbon source. The maximum of glycyrrhizin accumulation was obtained with 6% sucrose [(76.07 ± 6.82) $\mu\text{g/g}$ dry wt]. Gly-

cyrrhizin accumulation gradually decreased when 9% and 12% sucrose were applied (Fig. 3B). Maximum glycyrrhizin production per flask was also found with 6% sucrose [(28.78 ± 1.58) $\mu\text{g/flask}$] (Fig. 3C). These results suggested that 6% sucrose was optimal for growth and accumulation of glycyrrhizin in *G. inflata* hairy roots.

Production of secondary metabolites reflects the adaptations of plants to abiotic and biotic stress. Elicitors are widely used to enhance the produc-

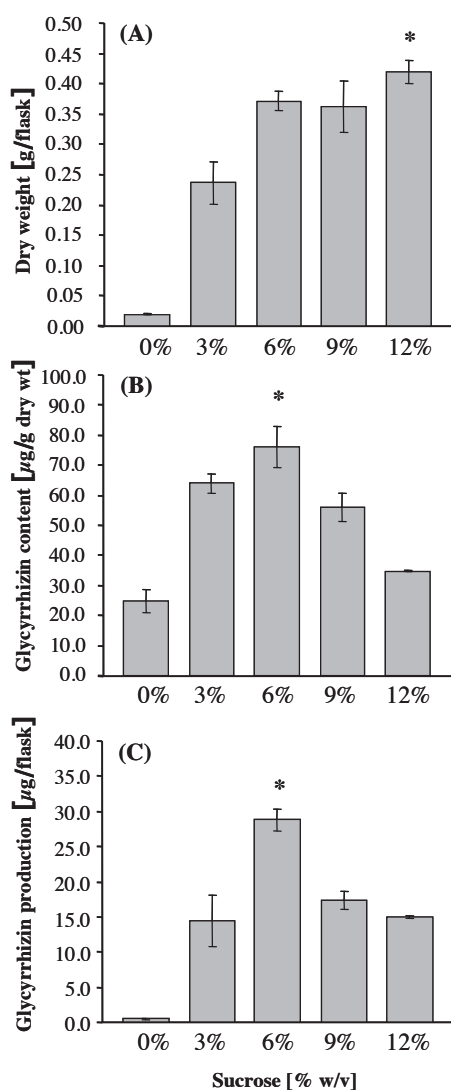


Fig. 3. Effects of sucrose on (A) biomass production, (B) glycyrrhizin content, and (C) glycyrrhizin production per flask in *G. inflata* hairy roots; * indicates statistical significances at $P < 0.01$.

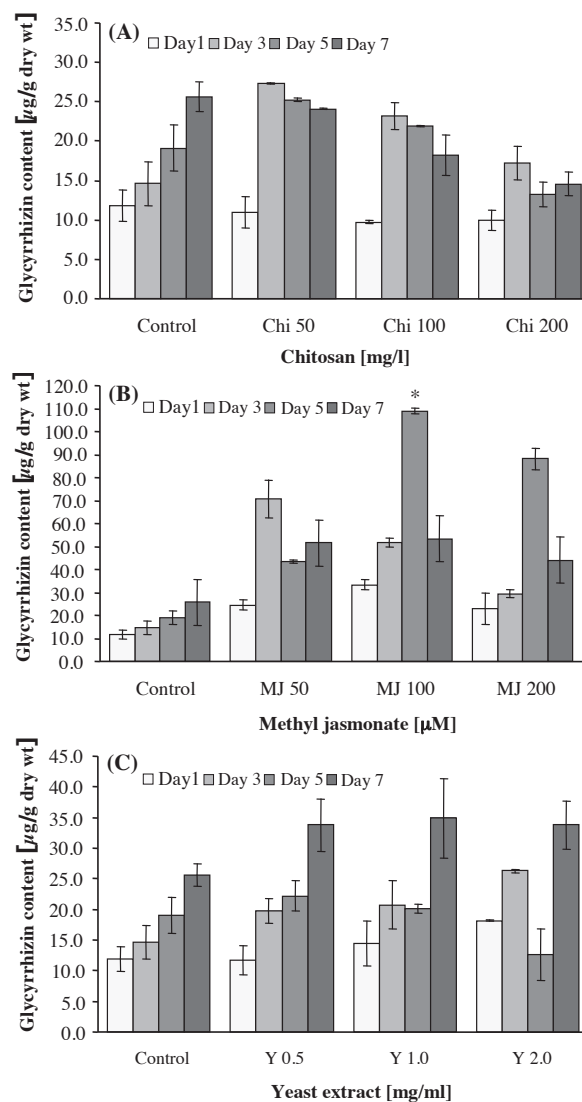


Fig. 4. Effects of (A) chitosan (Chi), (B) methyl jasmonate (MJ), and (C) yeast extract (Y) on glycyrrhizin content in *G. inflata* hairy roots; * indicates statistical significances at $P < 0.01$.

tion of such compounds in plant cell cultures. We have determined the effects of various elicitors at different concentrations on growth and glycyrrhizin production in *G. inflata* hairy roots. There was no significant difference in growth between the control and treated groups during elicitation, suggesting that the elicitors did not affect biomass production. As shown in Fig. 4A, chitosan treatment did not significantly change the content of glycyrrhizin. Moreover, glycyrrhizin contents decreased with the duration of the treatment. Thus, addition of chitosan is not suitable for enhanced glycyrrhizin production.

Fig. 4B shows the effect of methyl jasmonate on glycyrrhizin production in hairy roots. Glycyrrhizin production increased with the duration of the treatment (1–5 days) with 100 and 200 μM of methyl jasmonate. The highest glycyrrhizin accumulation was found after 5 days in samples treated with 100 μM methyl jasmonate [$(108.91 \pm 1.15) \mu\text{g/g}$ dry wt, 5.7 times higher than the control]. Further increase in the incubation period significantly reduced accumulation of glycyrrhizin. This may be due to the fact that longer elicitor contact leads to disturbances in cell permeability, osmotic condition, and changes in membrane potential (Vasconsuelo and Boland, 2007). These results indicate that methyl jasmonate was the most efficient elicitor for the enhancement of glycyrrhizin in hairy roots cultures of *G. inflata*. Our results reveal that exogenous application of methyl jasmonate to the hairy roots culture stimulates the production of glycyrrhizin. These results correspond to a previous study, which reported that

methyl jasmonate increased accumulation of glycyrrhizin in *G. glabra* whole plant cultures (Shabani *et al.*, 2009).

The effect of yeast extract on glycyrrhizin production in hairy roots is shown in Fig. 4C. Yeast extract showed a positive influence on glycyrrhizin production, but the yield was lower in comparison with methyl jasmonate. Glycyrrhizin accumulation was increased after adding 1.0 mg/ml yeast extract for 7 days [$(34.88 \pm 6.54) \mu\text{g/g}$ dry wt, 1.4 times higher than the control]. However, elicitation by the yeast extract did not show a significant difference between treated roots and control roots. Karwasara *et al.* (2010) reported that yeast extract (50 mg/l) added to *Abrus precatorius* cell cultures significantly stimulated the glycyrrhizin accumulation after treatment for 2 days. In our study, the effect of yeast extract on glycyrrhizin production was different from that in *A. precatorius*. This may be due to the difference in plant species, culture condition, and concentration of elicitor.

In summary, our results suggest that methyl jasmonate was the elicitor most effectively enhancing the level of glycyrrhizin. The present results might be useful for enhancing the accumulation of glycyrrhizin in hairy roots cultures of *G. inflata*.

Acknowledgements

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