Journal of Plant Biology, December 2005, 48(4) : 380-386

Growth Characteristics and Catalpol Production in Chinese Foxglove (*Rehmannia glutinosa* Liboschitz) Hairy Roots Transformed with *Agrobacterium rhizogenes* ATCC15834

Sung Jin Hwang*
Department of Oriental Medicine Materials, Dongshin University, Naju 520-714, Korea

Hairy root clones of *Rehmannia glutinosa* were established via transformation with *Agrobacterium rhizogenes* ATCC15834. To optimize the culturing conditions for both root growth and catalpol production, effects of various combinations of seven basal media, pH, and carbon sources were examined under darkness. The fastest root growth was obtained in an SH medium containing 4% sucrose (pH 5.8); the highest catalpol content (0.54% of dry weight) was achieved in a WPM medium supplemented with 4% sucrose (pH 5.8). Effects of plant growth regulators and chitosan were also investigated. IAA at 2 mg L^{-1} significantly increased root lengths and the frequency of lateral roots. Chitosan (50 mg L^{-1}) and GA_3 (0.5 mg L^{-1}) induced catalpol production, with contents calculated at 0.7% dry weight and 0.65% dry weight, respectively.

Keywords: catalpol, chitosan, hairy root cultures, PGRs, *Rehmannia glutinosa*

Chinese foxglove (*Rehmannia glutinosa* Liboschitz) is a perennial herb usually distributed in central China. It is the major ingredient in Chinese medicine for treating thirst disease, and is also prescribed as an anti-bacterial and anti-inflammatory agent. Chinese apothecaries use either fresh or dried roots. The major metabolites isolated from this plant are sterol, campesterol, catalpol, rehmannia, and some alkaloids (Takeatsu et al., 1996; Zhu, 1998). Iridoid glycoside exists in plants of many families and possesses a wide variety of biological properties, including purgative, liver-protective, anti-microbial, analgesic, anti-tumor, sedative, and anti-inflammatory activities (Ismailoglu et al., 2002). One example is catalpol, extracted from the foxglove roots.

Plant cell culture for the production of useful secondary metabolites has a number of advantages over conventional procedures, e.g., the assurance of a continuous supply of uniform-quality, highly specialized, natural components that cannot be produced in equal quality or specificity through biological means. Cell cultures also offer reliable quality control and availability independent of environmental changes. However, commercialization of the chemicals obtained depends mainly on identifying appropriate techniques for increasing productivity. For most plant species, the synthesis of secondary products can be enhanced by allowing the plant tissue to differentiate morphologically and form organs (Lindsey and Yeomann, 1983; Enders, 1994; Balandrin and Klock, 1998).

Studies on the calli from *R. glutinosa* have been published (Park et al., 1999) but an *Agrobacterium rhizogenes*-mediated transformation protocol for *R. glutinosa* has not previously been reported. Hairy root cultures represent an interesting alternative to de-differentiated cell cultures for the production of secondary metabolites. Because hairy roots originate from a single plant cell via infection with *A. rhizogenes*, they are usually considered genetically more stable than callus lines. Moreover, in contrast to de-differentiated cells, metabolite production from hairy roots is not repressed during the growth phase of the culture. Therefore, hairy roots usually produce secondary plant products without the loss of concentration that is frequently observed from callus or cell suspension cultures (Rhodes et al., 1990; Toivonen, 1993; Kittipongpatana et al., 1998).

Here, the establishment of transformed root cultures of *R. glutinosa* will be reported, and the effects of media type, pH, carbon source, plant growth regulators (PGRs), and chitosan on the development of hairy roots and the production of catalpol will be also reported.

*Corresponding author; fax +82-61-330-3309
e-mail botany@dsu.ac.kr

MATERIALS AND METHODS

Plant Materials

Seeds of *R. glutinosa* were surface-sterilized with 70% (v/v) ethanol for 5 min and 1% (v/v) sodium hypochlorite for 10 min. They were then rinsed four times in sterile distilled water and placed on a half-strength Murashige and Skoog (1962) medium (1/2 MS) in 110 × 20 mm Petri dishes. After the seeds were germinated under darkness at 25 ± 1°C, the plantlets were transferred to a solid MS medium containing 2% sucrose. They were maintained at 25°C under a light intensity of 54 μmol m^{-2} s^{-1} from cool white fluorescent lamps (16-h photoperiod).

Genetic Transformation and Selection of Root Clones

To induce hairy roots, leaves from *in vitro* grown seedlings were cut into small pieces and co-cultured with *A. rhizogenes* strain ATCC15834 for 36 h in the dark. The induced adventitious roots were excised and cultured on a 1/2 MS medium containing 300 mg L^{-1} cefotaxime to eliminate the bacteria. Five transformed root clones obtained after the excision of single roots were maintained and propagated in the dark on a hormone-free MS medium supplemented with 3% sucrose.

Culture of Hairy Root Clones

Seven different media were tested for their effects on biomass yield and catalpol production: 1/2 MS, full-strength MS, B5 (Gamborg et al., 1968), LS (Linsmaier and Skoog, 1965), SH (Schenk and Hildenbrandt, 1972), WPM (Lloyd and McCown, 1980), and NN (Nitsch and Nitsch, 1969). After autoclaving, the media pH was adjusted with 1N NaOH to a value of 4.8, 5.8, or 6.8. All experiments were carried out in 100-mL Erlenmeyer flasks containing 40 mL of the liquid medium, inoculated with ca. 0.05 g (fresh weight) tissue. Sub-culturing was done at three-week intervals. The flasks were incubated on a rotary shaker at 100 rpm at 25 ± 1°C under darkness. After 12 weeks of culture, biomass dry weights and catalpol contents were determined.

Treatment with Plant Growth Regulators

To select the optimal combination that supported hairy root growth and stimulated catalpol synthesis, effects of supplemental PGRs in basal media containing 4% sucrose were tested. PGRs were added after the media were sterilized with a 0.45 μm membrane filter.

Chitosan Treatment

Chitosan (Sigma, USA) was dissolved in 6% (w/v) acetic acid, and the insoluble materials were removed by centrifugation. The dissolved chitosan was precipitated by the addition of 5N NaOH, then recovered by centrifugation. This procedure was repeated twice. The chitosan residue was washed three times with distilled water and freeze-dried (Merkli et al., 1997). The final chitosan concentrations in the hairy root cultures ranged from 5 to 500 mg L^{-1}. Besides being a simple elicitor, chitosan permeabilizes plant cell and tissue cultures. Therefore, the chitosan-dependent change in catalpol concentration in the liquid media was also determined.

Sample Preparation and Analytical Methods

The content of catalpol, the predominant secondary metabolite from *R. glutinosa*, was determined according to the method of Park et al. (1990). Hairy roots were harvested and lyophilized to measure metabolite contents. Lyophilized samples (ca. 1 g dry weight) were crushed and extracted with 80% methanol. Following filtration through a 0.45 μm Millipore filter, the root extracts were subjected to HPLC analysis. The chromatographic separation was carried out on a Capcell pack C_{18} reverse-phase column; UV levels were detected at 218 nm. The mobile phase contained 1:100 (v:v) acetonitrile:water, at a flow rate of 1.2 ml min^{-1}. Catalpol concentrations were quantified with an external standard of catalpol (Wako, Japan). For fresh-weight determinations, the roots were gently pressed on filter papers to remove excess water and weighed. They were then freeze-dried for a minimum of 48 h at 10^{-1} mbar (-42°C) before their dry weights were recorded.

RESULTS AND DISCUSSION

Hairy root cultures of *R. glutinosa* were established by co-cultivating sterile leaf segments with *A. rhizogenes*. These roots were easily identified by their rapid, highly branching, plagiotropic pattern of growth, and usually covered the surface of the solid medium within 3 to 4 weeks (Fig. 1A and B-1). Hairy root clones, derived from a single hairy root mer-

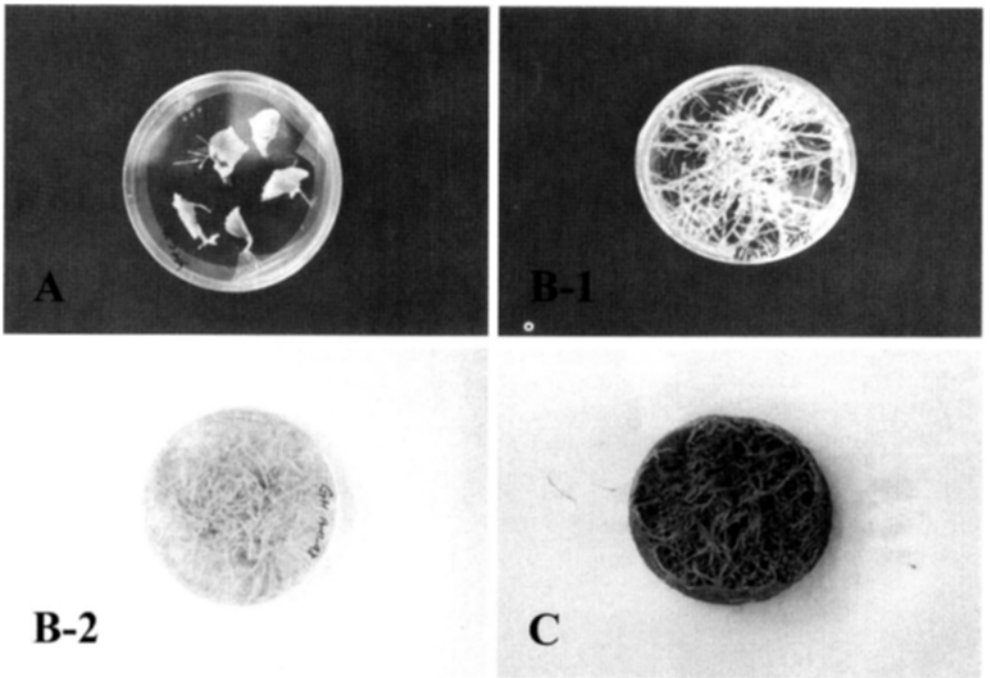

Figure 1. **(A)** Transformed roots induced from leaf explants by co-cultivating with *A. rhizogenes*. **(B)** Hairy root cultures on solid 1/2 medium (B-1) and SH medium (B-2) containing 4% sucrose. **(C)** Hairy roots cultivated in liquid WPM medium containing 4% sucrose, pH 5.8, after 12 weeks.

istem, may show variability in their branching frequency, growth rate, and production level (Mano et al., 1989; Bhadra et al., 1993). Here, 15 adventitious root clones based on the phenotypic characteristics of their roots on a solid medium with 2% sucrose were selected. The most actively growing strain RH 105 was isolated and used for determining the optimal culturing conditions.

Effects of Media, Carbon Source, and pH on Root Growth and Catalpol Biosynthesis

Manipulation of media components is an important strategy for improving the yield of useful secondary metabolites through cell and tissue culture (Ikeda et al., 1976; Mizukami et al., 1977; Wysokinska and Chmiel, 1997; Yamamoto and Kamura, 1997). Merkli et al. (1997) have reported that, for *Trigonella foenum-graecum* hairy root cultures, the fastest growth is obtained in a WPM medium, with the highest content of diosgenin being observed in a half-strength WPM medium. However, Ikenaga et al. (1995) have achieved optimal growth and steroidal saponin production in *Solanum aculeatissimum* hairy root cultures using a B5 medium containing 3% sucrose. On the growth of *R. glutinosa* hairy roots, the optimal performance, expressed as root dry weight per flask, was attained in the hormone-free SH medium (Fig. 1B-2 and 2). In contrast, neither RCM nor B5 medium were suitable for promoting the growth of hairy roots. Catalpol production, quantitated as % of dry weight, was also affected by media choice, with a high content being measured from tissues cultured in the hormone-free WPM medium (Fig. 1C, 2). Therefore, further experiments will be required to investigate the relationship between tissue growth and metabolite production as a function of media composition.

The effects of sucrose concentration on growth and the yield of secondary products have been examined in a number of plant tissue culture systems (Ikeda et al., 1976; Amorim et al., 1977; Sato et al., 1991). Transformed roots are sensitive to the combination of media components, especially mineral ions and carbon source, with regard to both growth and productivity (Wysokinska and Chmiel, 1997). Here, it was found that both growth and catalpol content were highest when the media were supplemented with 4% sucrose. These results are similar to those in hairy root cultures from other medicinal herbs (Bakkali et al., 1997; Giri et al., 1997; Liu et al., 1997). A high sucrose concentration is also necessary for promoting the high yield of shikonin derivatives in *Lithospermum*

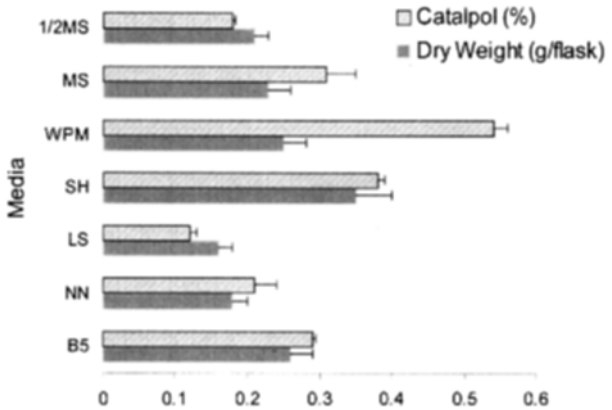

Figure 2. Effects of basal media on growth and catalpol content in hairy root cultures of *R. glutinosa*.

erythrorhizon, solasodine in *Solanum aviculare*, artemisinin in *Artemisia annua*, and diosgenin in *Dioscorea deltoides* (Mizukami et al., 1977; Battat et al., 1989; Liu et al., 1997; Kittipongpatana et al., 1998). In contrast, when the sucrose concentration exceeds 3%, growth can be inhibited and morphologies modified, with roots appearing callused and lacking lateral branching, probably due to osmotic stress (Hamill et al., 1986; Nguyen et al., 1992). In the present experiments, sucrose concentration over 10% retarded early development and caused prolonged root growth. Although both sucrose and glucose are widely used in plant tissue culture as carbon sources, transformed roots may show different growth rates in response to the available carbon, depending on their genotype. Jung et al. (1992) have proposed that catharanthine production in hairy root cultures of *Catharanthus roseus* can double when fructose is supplemented. However, the use of fructose also results in an approximately 40% decrease in root growth compared with the effect of sucrose. In the present study, growth and catalpol production were the highest when sucrose was supplied as the sole carbon source. All other sources, i.e., fructose, glucose, and lactose, had no positive effects on catalpol content (data not shown). These experimental results are in agreement with those reported by Zenk et al. (1975) for *Morinda citrifolia* cultures.

Although hairy root development was similar at pH 5.7 or 5.8, catalpol production was higher (0.54% of dry weight) at pH 5.8 (Fig. 4). Root growth was slightly inh ibited below pH 5.2 and above pH 6.2. In addition, it is noteworthy that in all cases, the media pH remained stable from the beginning to the end of the culture period. For *T. foenum-graecum* transformed root cultures, the effect of media pH is more pronounced for diosgenin content, but it does not influence root growth (Merkli et al., 1997). Liu et al. (1998) have also reported that the dry weights of hairy root cultures are almost constant for pH between 5.0 and 6.5, but the optimum for artemisinin accumulation was at pH between 5.7 and 5.8.

Effect of Exogenous PGRs on Growth and Catalpol Biosynthesis

The media for hairy root cultures normally do not require supplemental PGRs because the genes that

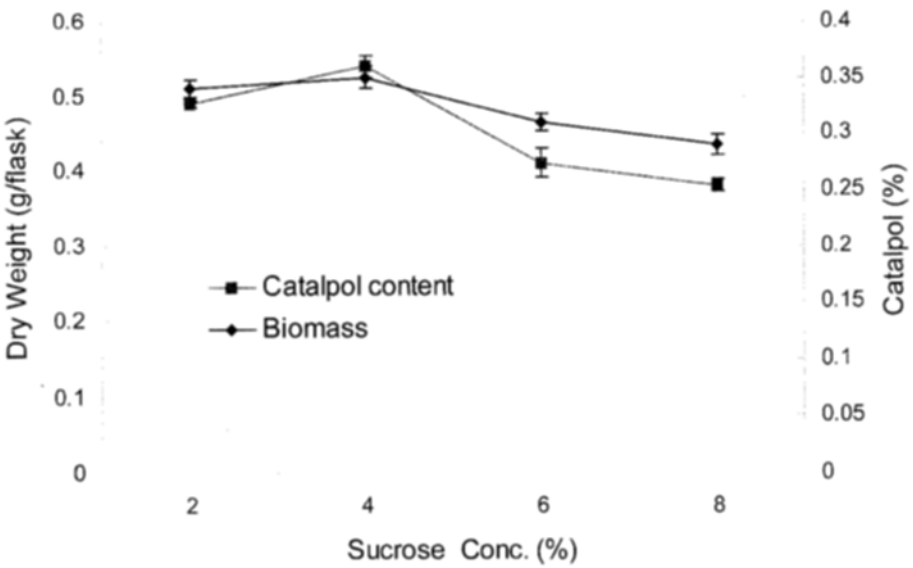

Figure 3. Effects of sucrose on growth and catalpol content in hairy root cultures of *R. glutinosa*.

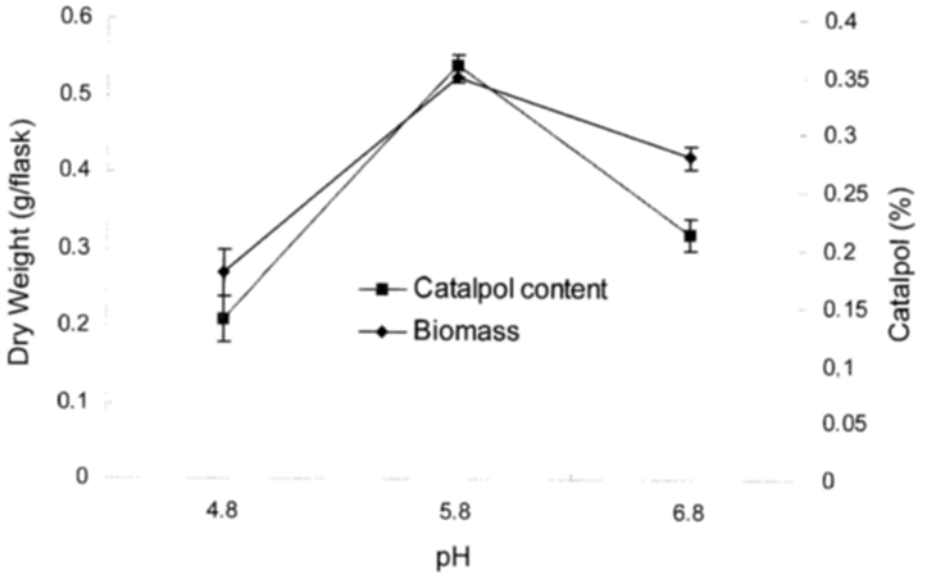

Figure 4. Effects of medium pH on growth and catalpol content in hairy root cultures of *R. glutinosa*.

increase sensitivity to auxin are present in the Ri plasmid. However, exogenous auxins or cytokinins can affect root growth and the formation of secondary products. This response might be related to insufficient synthesis of endogenous plant hormones in the transformed roots as well as the expression of *rol* genes (Wysokinska and Chmiel, 1997). In the present study, growth was most rapid in hairy root cultures of *R. glutinosa* when the basal medium contained 2 mg L^{-1} IAA (Table 1); IBA and NAA were less effective. However, the addition of cytokinin decreased hairy root growth and stimulated callus formation; 2,4-D and ABA also reduced biomass and catalpol production. The cytokinin-enhanced increase in root growth was the result of greater branching and root elongation rates. Similarly, Sato et al. (1991) have demonstrated with *Rubia tinctorum* that hairy roots cultured in the presence of IAA have maximal growth rates and the highest level of alkaloid production.

It has been reported that IAA and GA_3 supplementation to hairy root cultures of *Panax ginseng* improves growth ratios and metabolite contents, respectively (Hwang et al., 1999). Yamamoto and Kamura (1997) have also noted a five-fold increment in hairy root growth for *Bupleurum falcatum* within 8 weeks when propagated in an MS medium containing 5 μM IBA. Moreover, they have observed the formation of many lateral roots of normal appearance. However, Liu et al. (1997) have reported that root cultures of *Artemisia annua* have strongly inhibited root-tip elongation and a corresponding reduction in biomass. Rhodes et al. (1994) have shown that growing hairy root cultures of *Nicotiana rustica* in a basal medium containing auxin and low-level kinetin results in disorganized roots and a decrease in nicotin biosynthesis.

Table 1. Effects of PGRs on growth of *R. glutinosa* hairy root cultures.

PGRs (mg L^{-1})		Fresh weight (g/flask)	Dry weight (g/flask)
IAA	0.5	14.3±0.2	0.33±0.02
	1	14.7±0.2	0.38±0.03
	2	15.4±0.3	0.43±0.02
IBA	0.5	14.2±0.3	0.31±0.02
	1	14.1±0.4	0.26±0.03
	2	13.7±0.4	0.27±0.03
NAA	0.5	14.1±0.1	0.26±0.02
	1	14.3±0.3	0.31±0.03
	2	14.0±0.3	0.21±0.02
2,4-D	0.5	13.1±0.3	0.15±0.03
	1	12.6±0.4	0.13±0.04
	2	12.1±0.4	0.17±0.03

Experiment was done in duplicate. Mean±SD.

Hairy roots of *R. glutinosa* in the present study accumulated higher levels of catalpol when 0.5 mg L^{-1} GA_3 was added to the WPM medium (Table 2). These results are similar to those obtained for hyosyamine production in *Datura stramonium* and artemisinin production in *Artemisia annua* (Robins et al., 1991; Liu et al., 1997). However, in hairy root cultures of that latter species, GA greatly stimulates increase in fresh weight, mainly due to enhanced branching,

Table 2. Effects of PGRs on catalpol production in hairy root cultures of *R. glutinosa*.

PGRs (mg L^{-1})		Catalpol contents (% dry weight)
IAA	0.5	0.43±0.02
	1	0.48±0.02
	2	0.53±0.03
IBA	0.5	0.41±0.02
	1	0.36±0.02
	2	0.37±0.02
GA_3	0.5	0.65±0.02
	1	0.41±0.03
	2	0.32±0.03
BA	0.5	0.25±0.03
	1	0.21±0.03
	2	0.17±0.04

Experiment was done in duplicate. Mean±SD.

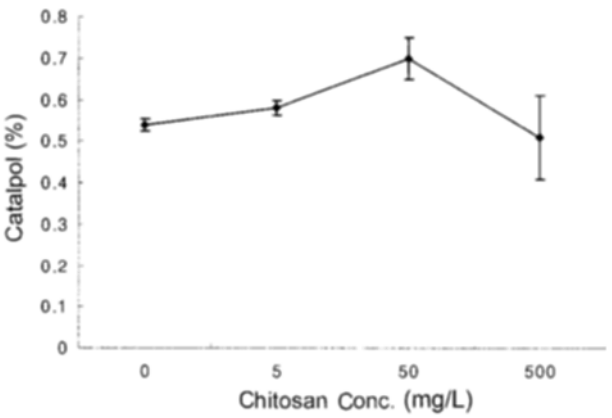

Figure 5. Effects of chitosan on growth and catalpol production in hairy root cultures of *R. glutinosa*.

while simultaneously inhibiting the production of total alkaloids (Liu et al., 1997). Therefore, the differing effects of PGRs on growth and secondary metabolite production are probably related to the genotype and physico-chemical characteristics of the explants.

Effects of Chitosan on Growth and Catalpol Biosynthesis

Chitosan, a polymer of beta-1,4-D-glucosamine, is obtained by alkaline hydrolysis of shellfish chitin. It is well known as an inducer of plant secondary metabolites (Funk and Brodelius, 1990). To test whether supplemental chitosan influences hairy root growth and catalpol accumulation, different concentrations of chitosan were added to basal media supplemented with 3% sucrose. After 12 weeks of culture, the additional chitosan had little effect on growth, but did cause increased catalpol production (Fig. 5). The medium supplemented with 50 mg L^{-1} chitosan showed the highest catalpol content (0.70% of dry weight), 1.3-fold greater than that measured in the control. However, this higher chitosan concentration also inhibited hairy root growth and, subsequently, led to reduced catalpol production. These results agree with those of Merkli et al. (1997), who demonstrated that culturing hairy roots of *T. foenum-graecum* in a medium containing 40 mg L^{-1} chitosan have little effect on growth when compared with the control, but significantly increase the diosgenin content. When the effect of various chitosan concentrations on the release of compounds was assayed, no catalpol was detected in any of the media.

Received June 15, 2005; accepted August 15, 2005.

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