Korean J. Medicinal Crop Sci. 12(5) : 378 - 383 (2004)

Effects of Elicitors on Scopolamine Production of *Scopolia parviflora* Nakai Adventitious Roots in Bubble Column Bioreactor

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ABSTRACT : Scopolamine and hyoscyamine are important anticholinergic compounds. To increase the productivity, we have selected various elicitors and developed culture system using a bubble column bioreactor (BCB). As the same manner of elicitation in flask cultures, the elicitors were introduced into BCB cultures and the productivity was investigated. Except the bacterial elicitor of *Staphyllococcus aureus*, the elicitors inhibited hyoscyamine production. In scopolamine production, the elicitors revealed different responses from the results obtained in flask cultures. The elicitors of KCl and *Candida albicans* less increased the production than flask cultures. However, methyl jasmonate and *S. aureus* showed stronger positive effects on tropane alkaloid production. In particular, *S. aureus* was the most effective elicitor on scopolamine production and the elicitor resulted in the highly increased production, approximately 10 times higher than the control culture.

Key words : *Scopolia parviflora*, *Staphyllococcus aureus*, bubble column bioreactor, elicitor, scopolamine

INTRODUCTION

Scopolamine and hyoscyamine are representative compounds of tropane alkaloids (TA). These are widely used in pharmaceutical industry in relation to the parasympathetic nervous system (Yamada and Tabata, 1997). Among the several korean native solanaceous plants producing TA, *Scopolia parviflora* is the only species that has ability to accumulate the compounds. The root of *S. parviflora* is well known as Nangtangeun in Oriental medicine, which was used as anticholinergic agent for a long time (Jung *et al.*, 2002).

The supply of TA is still dependently on direct extraction of cultivated plants due to the difficulty of artificial synthesis. The current supply system, howerer, is unstable because a the raw material production is dependent on the chialic conditions (Aoki *et al.*, 1997). Therefore, it is needed the establishment of *in vitro* culture for stable production and biotechnological strategies for enhancement of metabolite productivity. As the biosynthesis of TA is related with root differentiation (Endo & Yamada, 1985), many researchers have studied TA production with hairy and adventitious roots of *Atropa*, *Hyoscyamus*, *Duboisia*, and *Datura* species (Kamada *et al.*, 1986; Pitta-alvarez *et al.*, 2000; Zabetakis *et al.*, 1998). However, there still remains low productivity, and various skills such as elicitation, cell line selection, design of various bioreactor type, and precursor feeding are demanded to elevate accumulation of target substance.

We have already reported the successful establishment of adventitious root culture system of

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Received September 13, 2004 / Accepted October 13, 2004

S. parviflora (Jung *et al.*, 2002) and the selection of effective elicitors like fungal elicitor (Jung *et al.*, 2003a), bacterial elicitor (Jung *et al.*, 2003b) and signaling molecule (Kang *et al.*, 2004) for TA production. In addition, small scale bioreactor was developed with bubble column bioreactor (BCB) to scale up culture and the optimized culture conditions were determined (in submitted). We found that TA production increased in BCB cultures as compared with shake flask cultures. Therefore, in present study, each of selected elicitors were added to BCB cultures to increase TA productivity. It is investigated whether the elicitors are still fruitful not only in shake flasks but also in expanded bioreactor cultures. In addition, the most effective elicitor was determined here.

MATERIAL AND METHODS

Plant material and root culture

S. parviflora was provided from the National Arboretum of Korea. The adventitious roots of *S. parviflora* were induced from the rhizome of a mature plant. The induced adventitious roots were cut and proliferated in the 1/2B5 liquid medium supplemented with 3% (w/v) sucrose and 0.1 ㎎/ℓ IBA. The liquid cultures were maintained at 100 rpm and 25℃ under dark condition and subcultured every 4 weeks. As the results of optimization of culture condition, all experiments were carried out with B5 liquid medium containing 5% (w/v) sucrose and 0.1 ㎎/ℓ IBA (Jung *et al.*, 2002).

Preparation of BCB culture

The BCB was developed for adventitious root cultures of S. parviflora in this study (Fig. 1). The bioreactor consisted of culture chamber (7.5×25 ㎝), air inlet and outlet, sampling ports, accessory connector and air filter (0.2 ㎜ pore size, Sartorius, Germany). The connection line was made of silicon tube. The internal diameter of the bioreactor was 6.5 ㎝, and the working volume was 300 ㎖ For the BCB culture, air was supplied through a sparger at the bottom of the reactor passing an air filter. The BCB culture was initiated by pouring the medium and inoculating the roots. Fourteen-day-old adventitious roots were randomly cut into 1.5~2 ㎝ segments and 5 g fresh roots were inoculated into a 600 ㎖ bioreactor containing 300 ㎖ of medium. The bioreactor placed at 25℃ under dark condition.

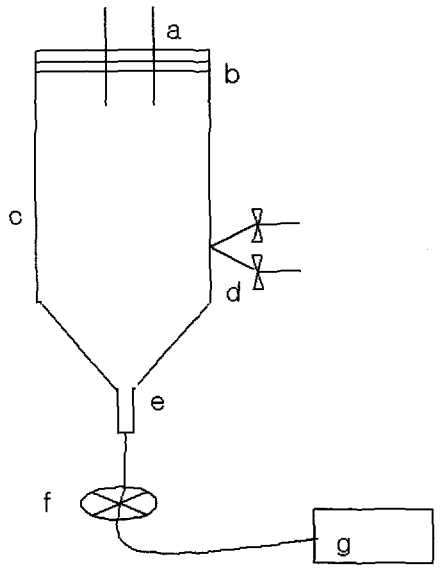

Fig. 1. A bubble column bioreactor (BCB) used in present study. The vessel volume was 600-㎖ (25× 7.5 ㎝) and the working volume was 300 ㎖. The glass growth chamber was connected to an air bubbler to provide oxygen. Supplied oxygen was sterilized by passing an air filter and bubbles were created through a sparger. a : stainless tube, b : stopper, c : culture vessel, d : sampling ports (not used in the present study), e : air inlet, f : air filter, g : air bubbler

Elicitation

NaCl and KCl inorganic salts were treated as abiotic elicitor. The stock solutions of NaCl and KCl were prepared with concentrations of 50, 200, and 800 mM, respectively. Each salt was dissolved in distilled water and sterilized by autoclaving at 121℃ for 15 min. Fourteen-day-old adventitious root cultures were fed with high concentration salts of NaCl and KCl. Methyl jasmonate (MJ), *Candida albicans*, and *Staphyllococcus aureus* elicitors were prepared as the same methods used for shake flask experiments, respectively (Kang *et al.*, 2004; Jung *et al.*, 2003a; Jung *et al.*, 2003b). On the basis of the results obtained in previous experiments, 800 mM KCl, 1.0 mM MJ, supernatant of C. albicans 0.07 ㎖/

㎖, and S. aureus 0.13 ㎖/㎖ were determined as the effective elicitors. The selected elicitors were incorporated into BCB cultures as the same methods in flask cultures. The elicitation effects were measured at 12, 24, 48 and 72 h after adding elicitors. The effect bacterial elicitor was investigated after further exposed by 144 h.

Measurement of root growth

The adventitious roots were separated from the medium, blotted and weighed. The growth was represented by a growth index (G.I.) which was calculated using the following equation.

$$\text{Growth index (G.I.)} = \frac{\text{Harvested fresh weight} - \text{Inoculated fresh weight}}{\text{Inoculated fresh weight}}$$

Quantification of TA

The cultures were quantified by high performance liquid chromatography (HPLC). Root samples were prepared by the protocol of Jung *et al.* (2002). A filtered sample was transferred to the HPLC employing a HPLC operating system (Gilson, France) equipped with a TSK gel ODS-80^{TM} column (4.6 ㎜×25 ㎝, 5 ㎛, Tosho) and a UV detector (Gilson, UV 3000) operating at a wavelength of 215 ㎚. The isocratic mobile phase was a mixture of CH_3CN and 50 mM K_2HPO_4 (22:78 v/v) adjusted to pH 3.0 with H_3PO_4. Quantification of TA was achieved by comparing the retention time with data obtained from the standards, and a co-chromatogram of the standards and samples.

Statistical analysis

Data were expressed as an average of three separate experiments. The statistical significance between contrasting treatments was assessed by Duncan's multiple range test ($p = 0.05$).

RESULTS AND DISCUSSION

Effect of high concentration of NaCl and KCl

Generally the treatment of NaCl and KCl was detrimental to root growth (data not shown). Treatments of both salts showed similar effects at the same treated concentration. Within 12 h after elicitation with 800 mM salts, the roots were severely inhibited and the root color turned into grayish at 24 h. Cell death occurred at 48 and 72 h at the concentration of 800 and 200 mM, respectively. However, slight increase of root growth was observed in both 50 mM NaCl and KCl. In appearance the roots were not affected by the concentration (mM) of both salts for 48 h after elicitation. Only at 72 h of exposure time, the roots began to be negatively affected in response to high concentration salts. Salt stress, in addition to the known factor of osmotic stress and ion toxicity, is also manifested as an oxidative stress, and all of these contribute to its deleterious effect (Gosset *et al.*, 1996). The adverse effect of NaCl has been attributed to changes in osmotic potential resulting from reduced water content. And also the effect may be due to specific toxic effects caused by the accumulation of sodium and chloride ions as observed in *Suaeda maritime, S. monoica* and other plants (Ali, 2000).

Salts induced the accumulation of the scopolamine, but not hyoscyamine (Fig. 2). In NaCl treatments, 200 mM NaCl was the most effective on the production of scopolamine at 24 h, and similar result was obtained in 50 mM NaCl at 12 h. However the highest concentration of NaCl (800 mM) did not affect on both scopolamine and hyoscyamine production for 48 h, and then the production suddenly decreased and was not detected after 72 h (Fig. 2A and B). There is a direct correlation between the induced activity of these and other specific enzymes and secondary metabolite accumulation. Although 800 mM of the salts severely inhibited root growth and hyoscyamine production, the maximum content of scopolamine was obtained in the treatment of 800 mM KCl, which was 2.3 times higher than the control (Fig. 2C). In hyoscyamine production, only the treatment of 50 mM KCl increased the hyoscyamine production at 48 h. The other treatments did not respond the elicitors of NaCl and KCl, and further the adventitious roots necrotized in the highest concentration of 800 mM NaCl and KCl (Fig. 2B and D).

Secondary metabolites in plant cell cultures accumulate in response to various agents termed abiotic elicitors. Inorganic salts are readily available, cheap and easy to use, and are also chemically defined.

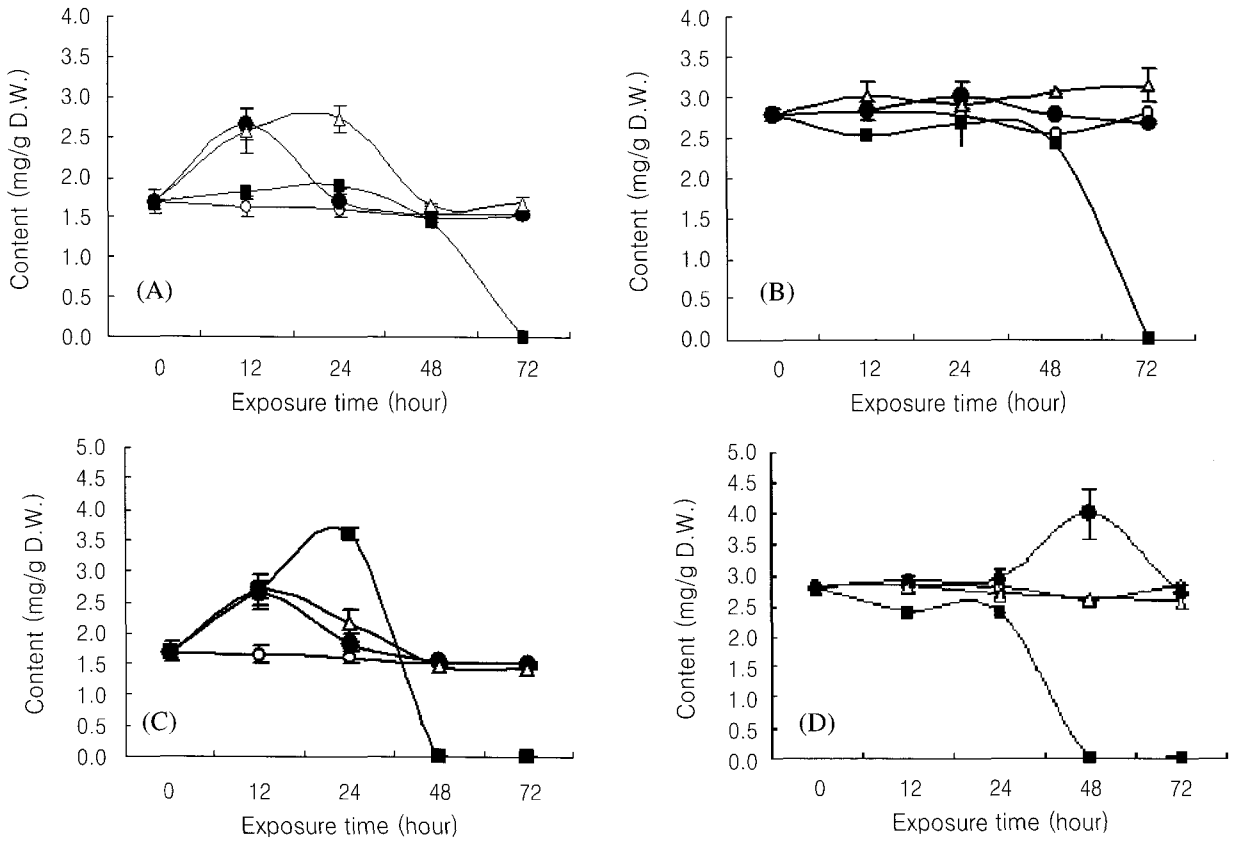

Fig. 2. Effects of various concentrations of NaCl (A and B) and KCl (C and D) on scopolamine (A and C) and hyoscyamine (B and D) production. Each value represents the mean and standard deviation (S.D.) of three replicates (○: control, ●: 50 mM, △: 200 mM, ■: 800 mM).

It has been proposed that the treatment of plant cell cultures with inorganic salts and heavy metals may be an ideal method of elicitation for commercial production of secondary metabolites and phytoalexins. It has been known that many enzymes involved in secondary metabolism can be regulated by several factors, such as Mg, Ca, and Na (Lee, 2002). PMT and/or H6H, key enzymes in scopolamine production, may be activated by addition of NaCl and KCl, and thus enhanced production of TA could be achieved.

Effect of signaling agents, fungal and bacterial elicitors

In the short run on the previous results of elicitation with MJ, MJ increased the scopolamine and hyoscyamine production at 1.0 mM of concentration and reached the maximum level after 12 h and 24 h, respectively (Kang *et al.*, 2004). In case of *C. albicans*, the supernatant elicitor was more effective on scopolamine production than the homogenate, and the production was increased up to 2.2 fold than the control at 24 h of elicitation with 0.07 mℓ/mℓ of concentration (Jung *et al.*, 2003a). The raw bacterial elicitor of *S. aureus* was proved as the best on the scopolamine production which was 2.8 fold higher compared with the control at 12 h with 0.13 mℓ/mℓ of concentration (Jung *et al.*, 2003b).

Effect of elicitation in BCB cultures

The elicitation increased the specific compound of scopolamine, while the production of hyoscyamine was slightly inhibited in BCB cultures (Table 1 and 2). The content of scopolamine was attained to the greatest level at 12 h of exposure time in response to KCl and MJ (Table 1). The culture treated with *C. albicans* produced the enhanced yield throughout 48 h and then slightly decreased. As compared with the

results obtained in flask cultures, the adventitious roots treated with KCl or *C. albicans* accumulated less scopolamine production. The result indicates that the content was elevated 1.3 and 1.7 fold higher than the control culture, with each addition of KCl and *C. albicans* into BCB cultures, while the each treatments showed more increased levels, 2.3 and 2.2 fold than the control in flask cultures. On the other hand, the individual cultures treated with MJ and *S. aureus* produced the enhanced yield of scopolamine from 1.3 and 2.8 fold in flask cultures to 2.1 and 9.9 fold in BCB cultures. These results might be due to the relatively different volumetric operation by scale up to bioreactors. And thus these quantitative increase including medium volume and added elicitors revealed different profiles of scopolamine production as compared with conical flasks.

In case of elicitation with 800 mM KCl, the less production of scopolamien in a BCB could be described in relation to polyamine metabolism. Because the biosynthetic routes of tropane alkaloids and polyamine are branched at putrescine, the report is important that the accumulation of putrescine occured in the leaves of K^+ deficient barley (Richards & Coleman, 1952). If the reversed result was obtained, the content of putrescine pooling to polyamine metabolism might be decreased by addition of exogenous K^+, and the metabolite flux might flow to tropane alkaloid metabolism. As a result, the increased metabolite flux might contribute to the elevated content of scopolamine.

Table 1. The effects of application of selected elicitors to a BCB on scopolamine production

Elicitors	Culture time (hour)						
	Control	12	24	48	72	96	144
KCl	1.7^{c*}	2.2^{a}	2.1^{ba}	1.9^{b}	0.0^{d}		
MJ	1.7^{cb}	3.5^{a}	2.1^{b}	1.6^{cb}	1.5^{c}		
C. albicans	2.0^{d}	2.1^{cd}	2.7^{b}	3.4^{a}	2.4^{cb}		
S. aureus	2.6^{c}	7.0^{e}	12.9^{d}	20.7^{c}	25.7^{a}	23.0^{b}	3.9^{f}

† Content is expressed as mg per g dry weight.
‡ Values bearing different letters in a line are significantly different at $p < 0.05$.

The best result was obtained by *S. aureus* (Table 1). The scopolamine content was dramatically increased for 72 h and reached the maximum yield, 25.7 mg/g (D.W.) (Table 1). The value was higher 9.9 times than control culture. Then, the content was considerably decreased and the root growth was also suppressed. The inhibition of root growth and metabolite production by the raw bacterial elicitor was less generated in BCB cultures. The adventitious roots vigorously grew throughout 72 h and cell death happened at 144 h of co-culture with the raw bacterial elicitor, whereas the cell death occurred within 48 h of elicitation in flask cultures. It might be due to relatively abundant oxygen dissolved in culture medium. In flask culture, the oxygen continuously decreased without additional supply of oxygen. On the other hand, in BCB culture limitation of oxygen less occurred by feeding oxygen from a bubbler. It might result in the delay of cell death.

So far the highest scopolamine content (32 mg/g D.W.) in hairy root culture of *Duboisia myoporoides* was achieved by repeated selection (Yukimune *et al.*, 1994). Although they reported more increased level of scopolamine than that in this study, long time of selection was required to obtain the value and further high content of the metabolite was associated with poor growth. On the other hand, the culture elicited with *S. aureus* produced 10 fold increment of scopolamine production within 3 days and the root still grew lively. Furthermore, bacterial elicitors have several advantages. The culture period of bacteria is shorter than that of fungi and the preparation of bacterial elicitor is simple as the elicitor is treated in state of raw entire culture without any process.

However, the hyoscyamine production was inhibited in elicited roots cultured in BCBs (Table 2). The

Table 2. The effects of application of selected elicitors to a BCB on hyoscyamine production

E Values	Culture time (hour)						
	Control	12	24	48	72	96	144
KCl	2.9[a]	2.5[b]	2.6[b]	2.4[b]	0.0[c]		
MJ	2.9[a]	2.4[b]	2.4[b]	2.4±0.0[b]	2.4[b]		
C. albicans	3.4[a]	2.8[b]	3.1[b]	2.9±0.1[b]	2.51[c]		
S. aureus	3.3[g]	3.6[cb]	3.7[cb]	3.8±0.1[b]	4.2[a]	3.7[cb]	2.5[d]

† Content is expressed as ㎎ per g dry weight.
‡ Values bearing different letters in a line are significantly different at $p < 0.05$.

treatment with *S. aureus* slightly increased 1.3 fold higher than the control culture. These results indicate the elicitor might selectively stimulate scopolamine production although hyoscyamine is a direct precursor of hyoscyamine. This suggest that complex mechanisms of elicitors and unknown factors might be involved in the scopolamine metabolism.

In present study, we selected the most effective elicitor of *S. aureus*, and found the important increment of scopolamine with treatment of the elicitor in addition to the elevated production obtained in BCB cultures. Thus, the mass production of scopolamine might be achieved by application of the raw bacterial elicitor of S. aureus.

LITERATURE CITED

Aoki J, Matsumoto H, Asako Y, Matsunaga Y, Shimomura K (1997) Variation of alkaloid productivity among several clones of hairy roots regenerated plants of *Atropa belladonna* with *Agrobacterium rhizogenes* 15834. Plant Cell Rep. 16:282-286.

Endo T, Yamada Y (1985) Alkaloid production in cultured roots of three species of *Duboisia*. Phytochemistry 24:1233-1236.

Jung HY, Kang MJ, Kang YM, Yun DJ, Bahk JD, Chung YG, Choi MS (2002) Optimal culture conditions and XAD resin on tropane alkaloid production in *Scopolia parviflora* hairy root cultures. Kor. J. Biotechnol. Bioeng. 17:525-530.

Jung HY, Kang SM, Kang YM, Kim YD, Yang JK, Chung YG, Choi MS (2003a) Selection of optimal biotic elicitor on tropane alkaloid production of hairy roots in *Scopolia parviflora* Nakai. Kor. J. Medicinal. Crop Sci. 11: 358-363.

Jung HY, Kang SM, Kang YM, Kang MJ, Yun DJ, Bahk JD, Yang JK, Choi MS (2003b) Enhanced production of scopolamine by bacterial elicitors in adventitious hairy root cultures of *Scopolia parviflora*. Enzyme Microb. Technol. 33: 987-990.

Kamada H, O kamura N, Satake M, Harada H, Shimomura K (1986) Alkaloid production by hairy root cultures in *Atropa belladonna*. Plant Cell Rep. 5:239-242.

Kang SM, Jung HY, Kang YM, Yun DJ, Bahk JD, Yang JK, Choi MS (2004) Effects of methyl jasmonate and salicylic acid on the production of tropane alkaloids and the expression of PMT and H6H in adventitious root cultures of *Scopolia parviflora*. Plant Sci. 166:745-751.

Pitta-alvarez SI, Spollansky TC, Giulietti AM (2000) Scopolamine and hyoscyamine production by hairy root cultures of *Brugmansia candida*: influence of calcium chloride, hemicellulase and theophylline. Biotechnol. Lett. 22:1653-1656.

Richards FJ, Coleman EG (1952) Occurrence of putrescine in potassium deficient barley. Nature 170:460-461.

Yamada Y, Tabata M (1997) Plant biotechnology of tropane alkaloids. Plant Biotech. 14:1-10.

Yukimune Y, Hara Y, Yamada Y (1994) Tropane alkaloid production in root cultures of *Duboisia myoporoides* obtained by repeated selection. Biosci. Biotech. Biochem. 58:1443-1446.

Zabetakis I, Edwards R, Hamilton JTG, O'Hagan D (1998) The biosynthetic relationship between littorine and hyoscyamine in transformed roots of *Datura stramonium*. Plant Cell Rep. 18:341-345.