Appl Microbiol Biotechnol (1992) 37:554–559

Applied and Microbiology Biotechnology

Scopolamine release into media by *Duboisia leichhardtii* hairy root clones

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Received 29 January 1992/Accepted 9 April 1992

Summary. The hairy root clones of *Duboisia leichhardtii* were found to release scopolamine into the medium. Among media examined, a modified Heller's medium that had 37 mM nitrate and no ammonium was suitable for scopolamine release. Scopolamine in the medium was efficiently recovered by the use of an Amberlite XAD-2 column. A reactor system consisting of a 2-l airlift reactor and a 25-ml column packed with Amberlite XAD-2 was constructed for production of scopolamine by the culture of the hairy root clone. The culture medium was passed through the column and the eluent from the column was back into the reactor continuously by using a low-pressure pump. When the hairy root clone DL47-1 was cultured in the reactor, 245 mg/l of scopolamine was released into the medium during 6 weeks and 97% scopolamine in the medium was recovered by the column. The scopolamine production was about five times higher in the column-combined reactor than in the reactor without the column. Scopolamine was recovered as the hydrobromide salt with more than 90% purity.

Introduction

Solanaceous plants such as *Nicotiana, Datura, Hyoscyamus, Scopolia* and *Duboisia* produce alkaloids including scopolamine and hyoscyamine. Many attempts have been made to produce alkaloids by culturing solanaceous plant cells (reviewed by Constable 1990). We reported that *Agrobacterium rhizogenes*-induced hairy root clones of *S. japonica* (Mano et al. 1986; Nabeshima et al. 1986) and *D. leichhardtii* (Mano et al. 1989) grew rapidly and produced tropane alkaloids such as hyoscyamine and scopolamine, although these tropane alkaloids were mostly retained in the root tissues.

Rhodes et al. (1986) reported that a culture of *N. rustica* hairy roots released 76% of the total nicotine into the medium. Also, it was reported that *Datura stramonium* hairy roots released small amounts of hyoscyamine and scopolamine (Robins et al. 1986) and *Duboisia leichhardtii* hairy roots also released a small amount of scopolamine into the culture medium (Mano et al. 1989).

The secondary metabolites released into the medium by a cell culture of *Cinchona ledgiriana* (Robins and Rhodes 1986), *Catharanthus roseus* (Payne et al. 1988) and hairy root culture of *N. rustica* (Rhodes et al. 1986) were recovered by the use of polymeric adsorbents. In these cases, the production of the metabolites was found to be increased by adsorption on a polymeric adsorbent column.

We here report selection of scopolamine-releasing hairy root clones of *D. leichhardtii*, the culture conditions suitable for scopolamine release, recovery of scopolamine from the culture medium with an adsorbent, and construction of a bioreactor system for continuous production of scopolamine by the culture of a hairy root clone.

Materials and methods

Chemicals. Scopolamine hydrobromide and L-hyoscyamine were purchased from Sigma (St. Louis, Mo., USA). Nicotine and ethylenediaminetetraacetic acid sodium iron (III) salt (FeNaEDTA) were obtained from Nakarai Tesque (Kyoto, Japan), Amberlite XAD-2, -4, -7 and -8 were from Organo (Osaka, Japan).

Culture media. Media used for the culture of hairy roots were: HF, Heller's medium (Heller 1953) with 0.078 mM FeNaEDTA instead of $FeCl_3 \cdot 6H_2O$; B5, Gamborg's B5 medium (Gamborg et al. 1968); SH, Schenk-Hildebrandt's medium (Schenk and Hildebrandt 1972); and SH-2, a modified SH medium with 0.216 mM FeNaEDTA instead of $FeSO_4 \cdot 7H_2O$ and Na_2EDTA, and with 2.61 mM $NaH_2PO_4 \cdot 2H_2O$ instead of $NH_4H_2PO_4$.

Hairy root cultures. Hairy root clones of *D. leichhardtii* were established as reported previously (Mano et al. 1989) and subcultured in HF liquid medium every 4 weeks. Approximately 100 mg

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fresh weight of each of hairy root clone was inoculated into 50 ml liquid medium in a 125-ml polycarbonate flask, and then incubated at 25° C in the dark on a model G10 gyratory shaker (New Brunswick Scientific, N. J., USA) at 85 rpm.

Adsorption and elution of alkaloids. Amberlite XAD resins were examined for adsorption of scopolamine in a culture medium. Resins were each washed in methanol twice prior to use, and then packed into 25-ml volume columns (diameter 20 mm × length 95 mm, Shibata-hario, Tokyo, Japan). The packed columns were each washed with 500 ml distilled water. A 500-ml volume of HF medium containing 200 mg/l of both scopolamine and hyoscyamine, and 2 mg/l of nicotine was passed through a resin column by using a low pressure pump (Master Flex, Cole-Parmer Instrument, Ill., USA) at a flow volume of SV 15. Then, the column was eluted with a 200 ml mixture of either ethanol and 28% NH_4OH (19:1 v/v) or methanol and 28% NH_4OH (19:1 v/v). Eluates were fractionated, passed through a preparatory filter (SJHV 004 NS, Nihon, Millipore, Tokyo, Japan) and then analysed by a HPLC with an octadecylsilica (ODS) column as reported previously (Mano et al. 1989).

Culture system. Hairy root clones were each cultured in a 2-l air-lift reactor (Shibata-hario) with and without a 25-ml volume column as shown in Fig. 1. Pretreated Amberlite XAD-2 was packed into the column. Then, both reactor and column were connected by a silicone tube (i.d. = 4 mm). A culture medium was passed through the column and the eluent was back into the reactor continuously by using a low pressure pump at a flow volume of SV 5. The column was removed every week and eluted with a mixture of methanol and 28% NH_4OH (19:1 v/v) to recover scopolamine. After washing with distilled water, the column was autoclaved and reconnected to the reactor. The reactor was aerated at a rate of 0.5 l/min by means of a glass filter at the bottom of the vessel. Influent air was passed through a 0.2-μm hydrophobic membrane filter (Millipore). The temperature was maintained at 25° C. The pH was monitored every 2 to 4 days.

Analysis of alkaloids. Alkaloids were extracted from cultured hairy roots by using a modified method of Hashimoto et al. (1986). Harvested hairy roots (approx. 50 mg dry weight) were freeze-dried, powdered by crushing in a test tube and then soaked overnight in a 5 ml mixture of ethanol and 28% NH_4OH (19:1 v/v). The extraction mixture was centrifuged for 10 min at 3000 rpm. Extraction with the basic alcohol was repeated twice, and combined extracts were evaporated at 42° C. Dried residues were dissolved in 1.0 ml of 0.1 M HCl, 0.9 ml of which was made alkaline with 0.2 ml of 1 M Na_2CO_3-$NaHCO_3$ buffer (pH 10.0). Then, 0.9 ml of an alkaline aqueous solution was loaded onto an Extrelut-1 column (Art. 15371, Merck, N.J., USA). After 5–10 min, 7.0–7.5 ml of $CHCl_3$ was applied to the column. The $CHCl_3$ extracts combined were dried at 36° C. Residues were dissolved in 0.1 ml of 70% ethanol and then analysed by HPLC with an ODS column as reported previously (Mano et al. 1989). In this procedure, the detection limit was 10 ng for both scopolamine and hyoscyamine, and 1 ng for nicotine.

Alkaloids were also extracted from a culture medium. One millilitre of a medium was made alkaline with 0.2 ml of the carbonate buffer and then treated as described above. Alkaloids adsorbed onto an Amberlite XAD-2 column were eluted with a 100 ml mixture of methanol and 28% NH_4OH (19:1 v/v). Then, 10 ml of the extracts were evaporated at 42° C. Dried residues were dissolved in 1.0 ml of 0.1 M HCl, made alkaline with 0.2 ml of the carbonate buffer and then treated as described above.

Purification of scopolamine. After running fermentation in the reactor system, the combined methanol/NH_4OH eluents from an Amberlite XAD-2 column were evaporated at 42° C. Dry residues containing approximately 450 mg scopolamine were dissolved in 40 ml of 0.25 M H_2SO_4. The acidic aqueous solution was filtered through filter paper no. 2 (Advantec Toyo, Tokyo, Japan) and then made alkaline with 5 ml of 28% NH_4OH. The solution was extracted twice with 40 ml $CHCl_3$ and the combined extracts were dried at 36° C. Residues were dissolved in 1.2 ml of 1 M hydrobromide. Insoluble residues including nicotine were removed by centrifugation for 5 min at 10000 rpm. After concentration of the supernatant, scopolamine hydrobromide was obtained as crystals.

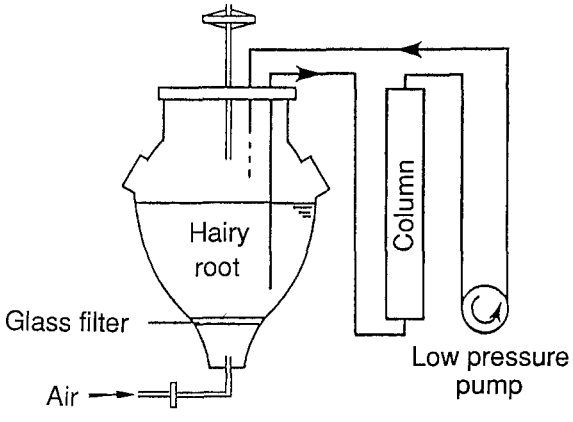

Fig. 1. A column-combined reactor system was constructed by the combination of a 2-l air-lift reactor and a 25-ml column packed with Amberlite XAD-2. The reactor was connected with the column by means of a silicone tube. Medium was passed through the column and eluent was back into the reactor continuously by using a low pressure pump

Results

Culture media for D. leichhardtii hairy root clones

The hairy root clones of *D. leichhardtii* released a small amount of scopolamine into the culture medium, as described in the previous report (Mano et al. 1989). Therefore, the most scopolamine-productive clone, DL-47, was cultured in each of SH-2, HF, SH, and B5 media for 4 weeks, and then analysed for scopolamine released into the culture media. As shown in Table 1, scopolamine was released into SH-2 to the highest degree, fol-

Table 1. Basal media used for the culture of the hairy root clone, DL-47, of *Duboisia leichhardtii*

Basal medium	Inorganic	nitrogen (mg/l)	NO_3^-/NH_4^+ (mM)	Scopolamine released (mg/50 ml medium)
SH-2	KNO_3	2500	24.8/0	0.30
HF	$NaNO_3$	600	7.0/0	0.20
SH	KNO_3	2500	24.8/2.6	0.05
	$NH_4H_2PO_4$	300		
B5	KNO_3	3000	29.7/2.0	N.D.
	$(NH_4)_2SO_4$	134		

Approximately 100 mg fresh weight of the hairy root clone DL-47 was cultured in 50 ml of each liquid medium (see Materials and methods). After 4 weeks of culture, scopolamine released into the medium was analysed as described in Materials and methods: N.D.; not detectable

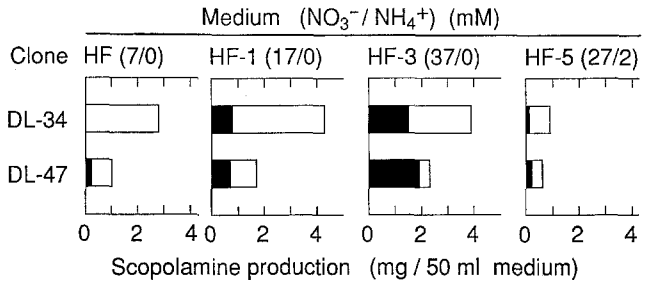

Fig. 2. Scopolamine release by the culture of *Duboisia leichhardtii* hairy root clones in HF medium (see Materials and methods) with various NO_3^-/NH_4^+ ratios. Two hairy root clones DL-34 and DL-47 were each cultured in the dark for 4 weeks at 25° C in 50 ml HF medium with various ratios of NO_3^- and NH_4^+. The *number in parenthesis* of each medium shows the concentration of both KNO_3 and NH_4Cl: ■, amount of scopalamine released into the medium; □, amount of scopolamine in root tissue

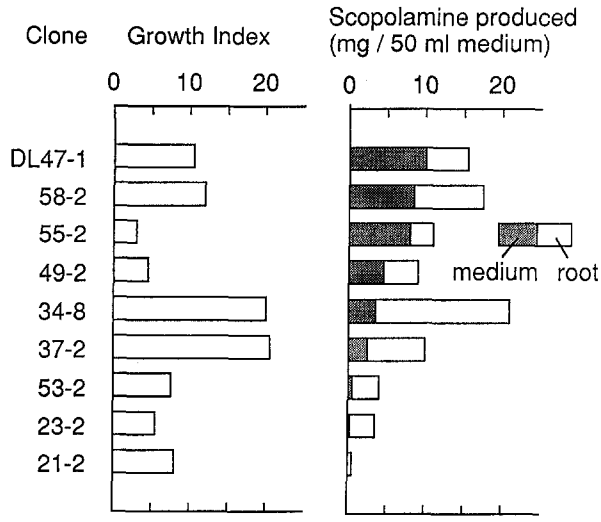

Fig. 3. Scopolamine release by the culture of the *Duboisia leichhardtii* hairy root clones in HF-3 medium. Forty-five hairy root clones were each cultured in the dark for 4 weeks at 25° C in 50 ml of HF-3 liquid medium. The results of nine representative clones are shown. The growth index is harvest dry weight (4 weeks) per inoculum dry weight. The scopolamine production column gives the amount of scopolamine in the medium (▬) and in the root tissue (□)

lowed by HF media. No scopolamine was found in B5 medium.

Since HF medium was suited for both growth and scopolamine production in the culture of *D. leichhardtii* hairy roots (Mano et al. 1989), scopolamine release was examined in the medium with various NO_3^-/NH_4^+ ratios. Two hairy root clones, DL-34 and 47, were cultured in HF medium with different ratios of NO_3^- and NH_4^+, for 4 weeks, and then scopolamine contents in both cultured roots and media were determined as shown in Fig. 2. Both clones produced a high amount of scopolamine in the media with high NO_3^- concentration and no NH_4^+, and released the highest amount of scopolamine into the medium with the NO_3^-/NH_4^+ ratio of 37/0 (HF-3 medium). Although the total amount of scopolamine produced was higher with DL-34 than with DL-47, scopolamine released in the medium was higher with DL-47 than with DL-34. The presence of NH_4^+ (2 mM) in the medium seemed to inhibit scopolamine production and release.

Selection of scopolamine-releasing hairy root clones

Approximately 50 hairy root clones of *D. leichhardtii* isolated previously (Mano et al. 1989) were reexamined for scopolamine release into HF-3 medium. After 4 weeks culture in HF-3 medium, growth index (harvest/inoculum), scopolamine contents in the cultured roots and the medium were determined. Figure 3 shows the results of nine representative clones. The highest amount of scopolamine was released into the medium with clone DL47-1 (subclone of DL-47), although the total amount of scopolamine produced was the highest with clone DL34-8 (subclone of DL-34). A small amount of nicotine and a negligible amount of hyoscyamine were also found in the medium (data not shown). The growth rate was the highest with clones 34-8 and 37-2. Growth index and scopolamine production of other clones ranged within those of the nine clones as shown in Fig. 3 (data not shown).

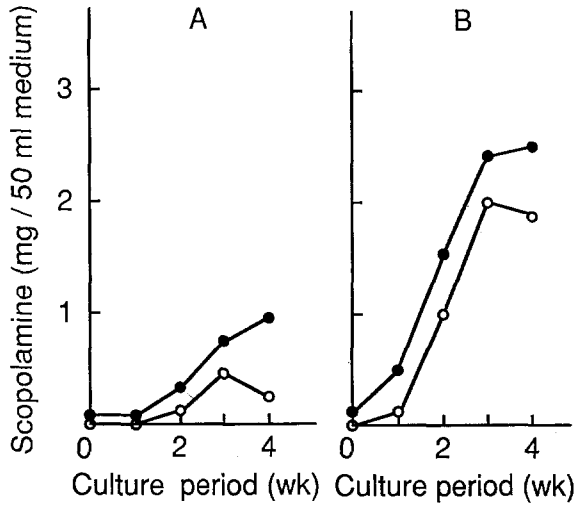

Fig. 4. Time course of scopolamine production in a culture of *D. leichhardtii* hairy root clone DL47-1 cultured for 4 weeks at 25° C in the dark in 50 ml of (**A**) HF and (**B**) HF-3 liquid media: ○, scopolamine released into the culture medium; ●, total scopolamine produced in the hairy root and medium

The time course in a batch culture of the hairy root clone DL47-1 was examined in 50 ml of both HF and HF-3 media as shown in Fig. 4. In HF medium, the total scopolamine produced was about 1.2 mg in 50 ml culture during 4 weeks and about half of the scopolamine was released into the medium. In HF-3 medium, the total scopolamine produced was 2.6 mg in 50 ml culture and about 75% was released into the medium, although the scopolamine contents in the cultured roots were nearly the same with both HF and HF-3 media. The re-

leased amounts for 4 weeks decreased in both HF and HF-3 media, although the total amounts increased. This may result from scopolamine degradation in the media or from adsorption of scopolamine into root tissues. Addition of a high concentration of KCl to HF medium showed no effect on scopolamine release (data not shown).

Recovery of scopolamine from the culture medium

Amberlite XAD resins were examined for adsorption of alkaloids in the medium. When 100 ml of HF medium containing scopolamine (200 mg/l), hyoscyamine (200 mg/l) and nicotine (2 mg/l) was applied to 25 ml of Amberlite XAD-2 column at flow volume SV 15, both scopolamine and hyoscyamine were adsorbed onto the resin, although nicotine and thiamine HCl (HF medium supplement) passed through the column. When another 100 ml of the medium was applied, 12% scopolamine and 3% hyoscyamine passed through the column, respectively. The adsorption amounts of these alkaloids (g/l resin) on Amberlite XAD-2, 4, -7 and -9 resins are listed in Table 2. Amberlite XAD-4, -7 and -9 adsorbed a larger amount of the alkaloids as compared with XAD-2. However, Amberlite XAD-2 was most selectively adsorbed both scopolamine and hyoscyamine. Diaion resins (Mitsubishi-kasei, Tokyo, Japan) also adsorbed fairly large amounts of the alkaloids, but not so selective for both scopolamine and hyoscyamine as Amberlite XAD resins (data not shown).

Recovery of the scopolamine adsorbed onto Amberlite XAD-2 resin was examined. A mixture of methanol and 28% NH_4OH (19:1 v/v) was suited for selective elution of scopolamine (data not shown). A double volume of the mixture was sufficient to elute 96% of the scopolamine from Amberlite XAD-2 (Table 2). The recovery from the other resins was lower than that from Amberlite XAD-2 under the same elution conditions. These results indicated that Amberlite XAD-2 was suited for selective adsorption and recovery of scopolamine from the medium.

Table 2. Adsorption and recovery of alkaloids from a culture medium by means of various adsorbents

Adsorbent	Adsorption (g/l-resin)[a]			Recovery (%)[b]
	Scop.	Hyos.	Nico.	Scop.
Amberlite XAD-2	1.1	1.9	0.02	96
4	3.5	>4.0	0.1	74
7	2.7	3.4	0.1	69
9	2.5	3.2	0.2	58

[a] HF medium containing 200 mg/l of both scopolamine (Scop.) and hyoscyamine (Hyos.), and 2 mg/l of nicotine (Nico.) was applied onto a resin column at flow volume SV 15. When 5% of the applied alkaloid was leaked from the column, the adsorption amount (g/l resin) was calculated

[b] Two volumes of a mixture of methanol and NH_4OH (19:1) was applied at a flow volume of SV 5 on each resin column. The recovery of scopolamine was measured

Table 3. Scopolamine production by the culture of the hairy root clone DL47-1 in a column-combined reactor

Location	Air-lift reactor without column	Column-combined reactor
	Growth index[a]	
	5.8 for 4 weeks	4.1 for 6 weeks
	Scopolamine (mg)	
Root (final)	28	74
Medium (final)	87	16
Recovered from column (wk)		
1	—	23
2	—	75
3	—	87
4	—	139
5	—	116
6	—	35
(subtotal)	—	475
Total	115	565

A 30 g fresh weight Sample of the hairy root clone DL47-1 was cultured in 2 l of HF-3 medium for 4 or 6 weeks

[a] The harvested fresh weight per inoculum fresh weight

Production of scopolamine by the culture of D. leichhardtii hairy root clone DL47-1

The production of scopolamine was examined by the culture of the scopolamine-releasing hairy root clone of *D. leichhardtii* in an air-lift reactor. The reactor system consisted of a 2-l air-lift reactor and a 25-ml volume of the column packed with Amberlite XAD-2 as shown in Fig. 1. When clone DL47-1 (30 g fresh weight) was cultured in the reactor without the column for 4 weeks, the growth index of the clone was 5.8. The total amount of scopolamine produced was 115 mg, of which 77% was released into the medium and 23% remained in the root tissue (Table 3). No more scopolamine was released into the medium during a further culture (data not shown).

In the column-combined reactor system, the clone DL47-1 was cultured for 6 weeks. The growth index was 4.1. During 6 weeks of culture, scopolamine released into the medium was continuously adsorbed onto the column. The total scopolamine produced was 565 mg, of which 13%, 3% and 84% were recovered in the root tissue, the medium and the column, respectively (Table 3). Therefore, 97% of scopolamine released in the medium was found to be recovered from the column. These results indicated that the culture of the scopolamine-releasing clone in the column-combined reactor produced about a five times larger amount of scopolamine than the culture in the reactor without the column.

Purification of scopolamine

After running a fermentation in the column-combined reactor system, scopolamine adsorbed onto the Amber-

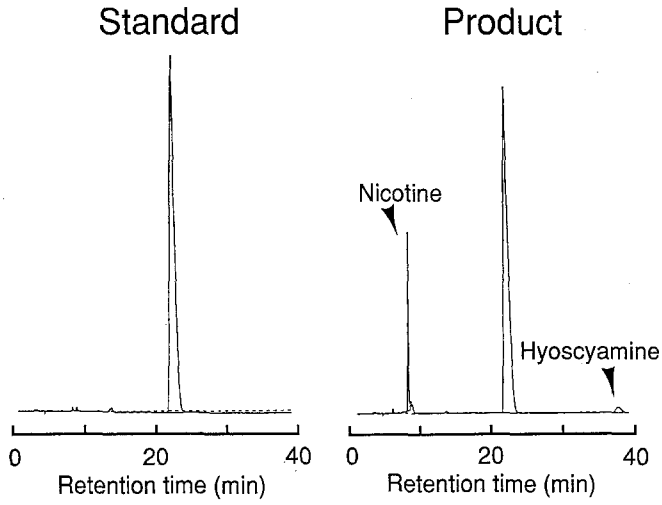

Fig. 5. HPLC analysis of scopolamine samples. Clone DL47-1 was cultured in a column-combined reactor for 6 weeks. Scopolamine adsorbed onto an Amberlite XAD-2 column was eluted, purified as HBr salts and analysed by HPLC as described in Materials and methods

lite XAD-2 column was eluted with a mixture of methanol and NH_4OH (19:1). Th eluted fraction was neutralized with 1 M hydrobromide to yield scopolamine hydrobromide. The yield of scopolamine hydrobromide was 84.6% on the basis of the product absorbed onto the resin column. The product was analysed by HPLC as shown in Fig. 5. The retention time was identical with that of authentic scopolamine. Also, the purified scopolamine sample was found to contain a small amount of nicotine and hyoscyamine. Thus, the purity of the purified sample was 90.3%. The structure of the product was confirmed by high resolution ^{1}H-nuclear magnetic resonance (data not shown).

Discussion

Endo and Yamada (1985) reported that the root culture of *D. leichhardtii* released scopolamine into the medium at a higher level than other *Duboisia* species such as *D. myopoloides* and *D. hopwoody*. In the present study, we selected the scopolamine-releasing clone from the *A. rhizogenes*-induced hairy root clones of *D. leichhardtii*. The clones obtained specifically released scopolamine into the modified HF medium. The *A. rhizogenes*-induced hairy root clones of *S. japonica,* which produced tropane alkaloids, released a negligible amount of scopolamine in the medium (Mano et al. 1986). Robins et al. (1986) reported that the hairy roots of *Datura stramonium* released hyoscyamine and scopolamine into the medium. However, the amount of scopolamine released from the clone DL47-1 of *Duboisia leichhardtii* was extremely high compared with the results of these previous reports.

Maximization of medium conditions for scopolamine release was also examined. Among the vairous modified HF media, HF-3 (37 mM of KNO_3 and no NH_4Cl) was the most suitable one for scopolamine release. A high concentration of NO_3^- may have some effects on the physiological conditions of the root tissue, including the changes of a membrane transport system as found with the cultured cells of *Thalictrum minus* (Yamamoto et al. 1987).

Payne and Shuler (1988) reported that *Vinca* alkaloids such as ajmalicine, ajmaline and yohinbine were selectively adsorbed onto Amberlite XAD-7. In the present study, Amberlite XAD-2 was suited for selective adsorption and recovery of scopolamine. With this resin, the adsorbed amount of nicotine was less than 2%. Since it was rather difficult to remove nicotine from scopolamine during the processes of purification and crystallization, the use of Amberlite XAD-2 may have an advantage for the purification process of scopolamine.

The production of scopolamine by a culture of the scopolamine-releasing hairy root clone DL47-1 in the column-combined reactor was much higher than that in the reactor without the resin column. During 6 weeks of culture, scopolamine released into the medium was continuously removed from the medium by the Amberlite XAD-2 column to reduce the scopolamine concentration in the culture medium. This may result in the suppression of feedback inhibition of scopolamine biosynthesis in the hairy root tissues and/or the reduction of scopolamine degradation in the medium.

It was reported that direct addition of an adsorbent into the culture medium enhanced the production of anthraquinone by a cell culture of *Cinchona ledgeriana* (Robins et al. 1986), indole alkaloids by a cell culture of *Catharanthus roseus* (Payne et al. 1988; Asada and Shuler 1989), and also nicotine by a hairy root culture of *Nicotiana rustica* (Rhodes et al. 1986). In the present study, we used a column containing the adsorbent. The metabolite scopolamine was continuously trapped onto the column and the hairy root clone was cultured for a long period with this system. As a result, the production of scopolamine was increased to about a five times higher amount as compared to the reactor without the column. To increase the productivity of scopolamine, further modification of a reactor system is now being examined.

Acknowledgement. The authors thank to Mrs. K. Kobayashi for technical assistance.

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