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Production of Tropane Alkaloids by Optimal Culture Conditions in Adventitious Root of *Hyoscyamus niger* L.

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ABSTRACT : Scopolamine and hyoscyamine are important anticholinergic compounds obtained from *Hyoscyamus niger*. Adventitious roots induced from rhizome of *H. niger* and roots were cultured in SH medium supplemented with 3% (w/v) sucrose and 0.5 ㎎/L IBA. Roots were grown rapidly after 10 days of cultures. Scopolamine production was increased 7 times and hyoscyamine production was increased 12 times after 10 day of cultures. SH medium was best in root growth. But, highest scopolamine productivity was observed in WPM medium, followed White medium and best hyoscyamine productivity was resulted in MS medium. Sucrose was increased scopolamine and hyoscyamine production were increased the medium supplemented by sucrose comparing to than those by other carbon sources.

Key words **:** *Hyoscyamus niger* L., adventitious roots, scopolamine, hyoscyamine

INTRODUCTION

H. niger, native to Scandinavia and southern England to the Mediterranean and northern Africa, is an annual herb of the family Solanaceae. This plant is coarse, foul-smelling, and very hazardous weed with all parts being poisonous. Despite this plant's weedy tendency and poisonous nature, it has great historical significance. Today, it has been cultivated as an ornamental plant and a crop for drug companies worldwide, especially United State and India.

Various tropane alkaloids (TA) have been used as pharmaceuticals and most of them are plant metabolites. The typical TA, atropine, hyoscyamine, scopolamine and cocaine were widely used as blockers of the parasympathetic nervous system such as anodyne and antispasmodic (Yamada and Tabata, 1997). However, these compounds are supplied by direct extraction of several Solanaceae plants such as *Atropa, Datura, Duboisia* and *Hyoscyamus* species. Thus the current supply method is problematic due to the limitation of cultivated regions and fluctuation in market price by unstable supply (Aoki *et al.*, 1997). Furthermore, the supplement of TA is entirely dependent on import in Korea.

An alternative to their extraction from plant tissue could be the uses of cell cultures. However it has been shown that TA biosynthesis is correlated with root differentiation. The use of undifferentiated cell cultures for the production of TA has proved unsuccessful because these compounds are synthesized in the roots (Endo and Yamada, 1985). For this reason, adventitious hairy root cultures offer an interesting approach to produce these secondary metabolites. Adventitious and hairy root cultures are a potential means for producing valuable plant compounds due to their fast growth rates and stable metabolite productivity (Carvalho and Curtis, 1998). A number of chemical and physical factors affecting cultivation have been tested extensively with various plant cells. Since there are many reports and patents concerning optimization of cultural conditions in order to improve growth rates of hairy roots and/or higher yield of desirable products (Jung *et al.*, 2002; Kang *et al.*, 2004). This study was carried out to investigate for optimal culture condition and pattern of growth and TA product to increase TA production in adventitious root cultures of *H. niger*.

MATERIALS AND METHODS

Plant material

Plants of *H. niger* were provided from Korea National Arboretum (Kwangnung, Korea) for *in vitro* cultures. Rhizome of *H. niger* were washed by flowed water using detergent enough, prior to surface sterilizing with 70% (v/v) ethanol for 1 min, 3% (v/v) NaClO for 10 min followed by rinsing in ster-

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ile water 5 times. Sterilized rhizomes were transferred to SH (Schenk and Hildebrandt, 1972) medium supplemented with 3% (w/v) sucrose, 0.5 ㎎/L IBA for induction of adventitious root. The medium was gelled with 0.38% gelite after adjusting pH to 5.7 and sterilized by autoclaving at 121℃ for 15 min. The cultures of rhizomes were incubated at 25℃ under dark.

Optimal culture condition for producing of TA

For determination of optimal medium in root growth and TA production, various media as MS (Murashige and Skoog, 1962), B5 (Gamborg *et al.*, 1968), WPM (Lloyd and McCown, 1980), LP (Quoirin and Lepoivre, 1977), SH, White (White, 1963) and NN (Nitsch and Nitsch, 1969) were tested. The 0.5 g of roots were inoculated into 100 mL Erlenmeyer flask contained 30 mL of liquid culture media, and were incubated at 25℃ under dark.

In order to identify the optimal carbon sources for TA production, with SH medium supplemented with 0.5 ㎎/L IBA and various concentrations (1%, 3%, 5% and 9%, w/v) of several carbon sources, such as sucrose, glucose and fructose were tested. The 0.5 g of roots were inoculated into 100 mL Erlenmeyer flask contained 30 mL culture media, and were incubated at 25 ℃ under dark. All culture of experiments were incubated within 4 weeks, and then each capability of growth and TA production was yielded.

Additionally, patterns of root growth and TA contents were investigated during 30 days with 5 days interval except first 2 days. TA contents were measured not only in roots, but also in medium.

Extraction and quantitative analysis of TA

TA content was determined using the method of Jung *et al.* (2002). Samples of the roots were prepared by extracting 1 g (F.W.) of adventitious roots with 10 mL mixture of 28% NH_4OH and 95% EtOH (1:19, v/v) for 2 h in ultrasonicator. The extracts were then centrifuged at 6,000 rpm for 10 min, and the supernatant solution was concentrated using a rotary vacuum evaporator. TA in the culture medium was directly extracted with chloroform in equal volumes by intermittent vortexing over 2 days. The combined chloroform phase was dried using the rotary vacuum evaporator. The residue was dissolved in 400 μL MeOH (HPLC grade), filtered through a prefilter (ψ0.2 μm Supelco) and analyzed by HPLC for quantitative determination of TA.

A filtered sample was analyzed by the HPLC employing a HPLC operating system (Gilson, France) equipped with a TSK gel ODS-80^{TM} (4.6 ㎜×25 ㎝, 5 μm, Tosoh) column and a UV detector (Gilson, UV 3000) 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 pH to 3.0 with H_3PO_4. After the injection of 20 μL of the sample solution, the column was operated at a flow rate of 0.8 mL/min. For quantitative analysis, the system was calibrated with standards of scopolamine and hyoscyamine purchased from Sigma. The correlation coefficients (R) of the standards were 99.9% for scopolamine and 99.7% for hyoscyamine. Quantification of TA was achieved by comparison with the retention time and co-chromatogram of the standards and samples.

Statistical analysis

Data were expressed as average of three separate experiments. The error bars indicate standard deviation (SD) from the mean of each replicate treatment.

RESULTS AND DISCUSSION

Effects of culture media

For determination of optimal medium on the *H. niger* adventitious root growth and TA production, several culture media were tested for 4 weeks (Fig. 1). Root growth was best in SH medium and followed by B5, NN and WPM medium.

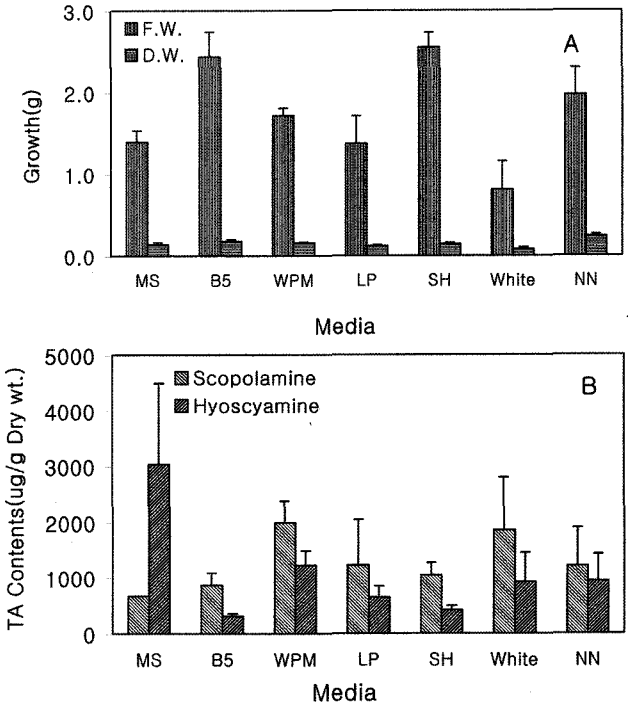

Fig. 1. Effect of culture media on growth and production of tropane alkaloids in adventitious roots. The 0.5 g of adventitious roots were cultured for 4 weeks in 100 mL flask containing 30 mL of various culture media. Each value is the mean of three replicates. Error bar means standard deviation (SD). (A) Growth of adventitious roots. (B) Contents of tropane alkaloids.

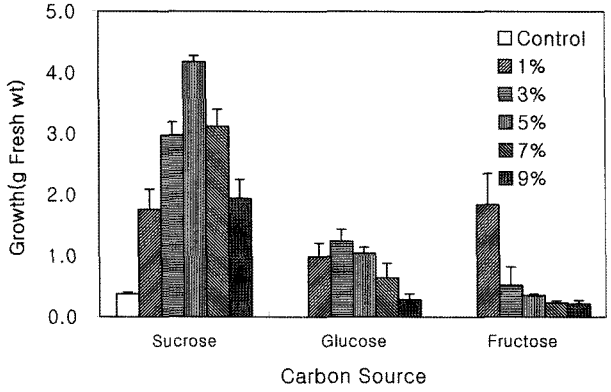

Fig. 2. Effect of various carbon sources on growth in adventitious roots. The 0.5 g of adventitious roots were cultured for 4 weeks in 100 mL flask containing 30 mL of SH medium supplemented 0.5 mg/L IBA, each carbon sources. Each value is the mean of three replicates. Error bar means standard deviation (SD). Control is the treatment un-supplemented carbon source.

However, White, LP and MS medium resulted in poor root growth. The TA production was more scopolamine abundant than that of hyoscyamine in all tested media except MS medium. The production of hyoscyamine was high in order of MS, WPM, NN, White, LP and SH medium. But, scopolamine was produced with the maximum yield in WPM medium, whereas the minimum was obtained in MS and B5 medium. In some cases, secondary product accumulation seems to be affected by culture medium (Biondi *et al.*, 2004). Cell suspension cultures of a boraginaceous herb *Lithospermum erythrorhizon* Sieb. et Zucc., which are incapable of synthesizing shikonin derivatives in LS liquid medium are known to produce a large amount of these compounds when transferred to M9 liquid medium (Fujita *et al.*, 1981).

Effects of carbon sources

The root growth and TA production were influenced by carbon source (Fig. 2). Especially, root growth was inhibited excepting sucrose treatment. The glucose and fructose containing cultures could not grow and cell death occurred.

Scopolamine and hyoscyamine were detected in all treatments (Fig. 3). The root growth and the hyoscyamine production were also enhanced by increasing concentration of sucrose. The production of hyoscyamine further augmented up to 9% of sucrose. The production of scopolamine was high at the 3%, and followed by 1% and 9% of sucrose. But, TA productivity in sucrose was worse than in other carbon sources. Although, glucose and fructose were poor in growth, TA productivity in these carbon sources was better than in sucrose. TA production in glucose and fructose has similar pattern through concentrations of carbon source.

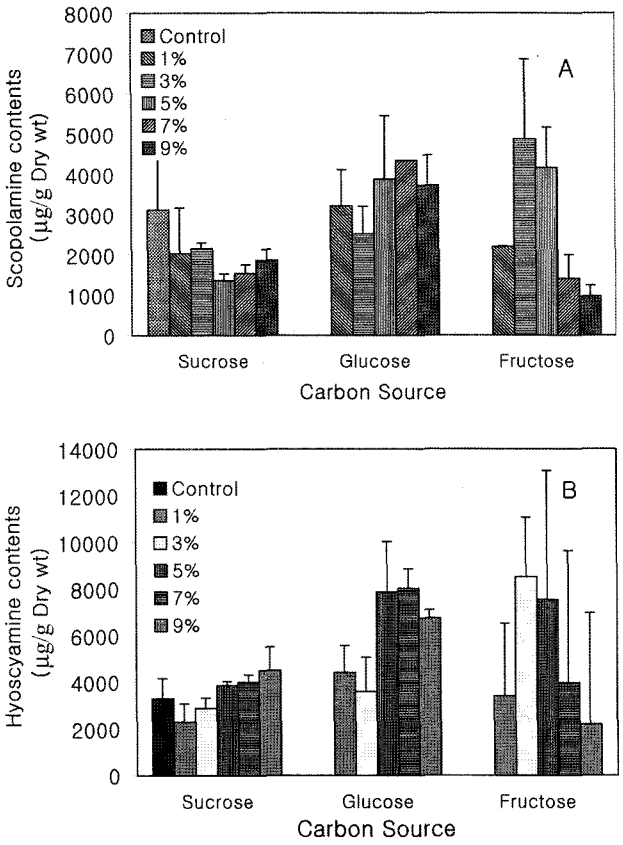

Fig. 3. Effect of various carbon sources on production of tropane alkaloids in adventitious roots. Each value is the mean of three replicates. Error bar means standard deviation (SD). (A) Contents of scopolamine. (B) Contents of hyoscyamine. Control is the treatment un-supplemented carbon source.

The relatively poor growth in the lowest and highest concentrations of sucrose may be resulted from difference of osmotic pressure. In addition, high concentration of sucrose produced high yield of secondary metabolite, and this result agreed to the previous report of Zhao *et al.* (2001). Takayama and Inoue (1997) reported that TA production was inhibited by increasing the osmotic potential of culture medium.

Growth and production profile of adventitious roots

Adventitious roots of *H. niger* were grew rapidly after 10 days of cultures and increased 8-folds after 4 weeks of cultures. The volume of cultured medium decreased throughout the culture periods (Fig. 4). In particular, the volume of cultured medium was considerably consumed after 30 days of cultures. The great consumption of the medium at the terminal stage between 25 and 30 days could be demonstrated in relation to the recovery of root growth.

The production profiles of scopolamine and hyoscyamine were similar in both on the basis of 1 g (D.W.) of adventitious

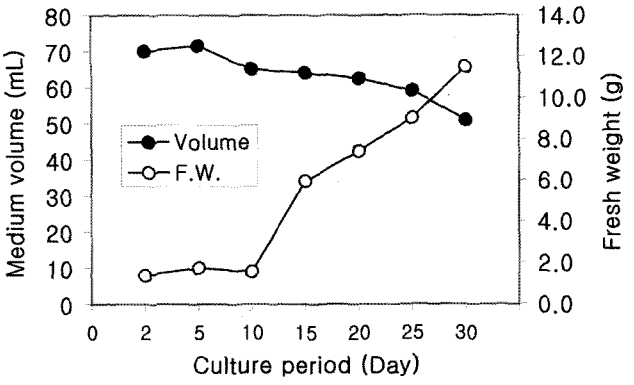

Fig. 4. Growth pattern of adventitious roots. The 1 g (F.W.) of roots were cultured on SH medium contained 3% sucrose, 0.5 mg/L IBA for 30 days.

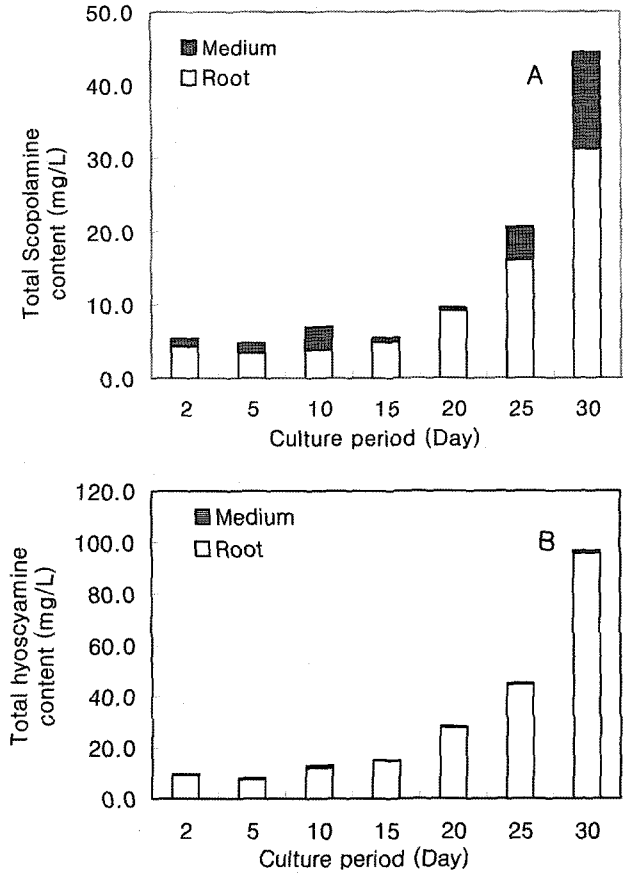

Fig. 5. Production pattern of tropane alkaloids in adventitious root cultures. (A) Total contents of scopolamine. (B) Total contents of hyoscyamine.

roots and total biomass (Fig. 5). The production of hyoscyamine was approximately 2 times higher than that of scopolamine. The production of scopolamine and hyoscyamine were increased by progression of the cultural periods. The relatively constant production was achieved on the basis of 1 g (D.W.) of adventitious roots throughout a cultural period, and thus the total production profile was identical with the growth profile.

Scopolamine and hyoscyamine were accumulated at the later stages of the growth cycle. As shown in Fig. 5, the excretion of TA into the culture medium was markedly increased by passing culture periods and the maximum excretion of TA gave after 30 days of exposure. Excretion of scopolamine in the culture medium was high compared to hyoscyamine.

Hairy roots usually store secondary metabolites in vacuoles inside the cells. Therefore, several methods have been used to increase the amount of products released into the medium (Wysokinska and Chmiel, 2004). Unfortunately, no general procedure is known that works in all cases, and the excretion behavior of hairy root cultures varies from one species to another, even within one species from one clone to another. However, The excretion of these compounds to the liquid medium offers possibilities for continuous cultures.

This study was determined culture conditions such as culture medium and the carbon sources for *H. niger* adventitious root cultures. In conclusion, TA production could be enhanced by the optimization of culture condition. These results could be applied successfully to large-scale production of TA by *H. niger* adventitious root cultures.

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