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Production of Tropane Alkaloids by Two-stage Culture of *Scopolia parviflora* Nakai Adventitious Root

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ABSTRACT : *Scopolia parviflora* Nakai, a rare and endangered species, is the sole plant producing tropane alkaloids (TA) among the Korean native species. In order to enhance TA productivity the SP72 root line was selected by screening 100 of root line, and the optimal culture media for root growth and TA production were investigated with the SP72 roots. Based on the several media, SH and 2B5 medium were determined as growth medium and White and NN medium as production medium. Among the four combinations of two-stage culture, 2BN (2B5 as growth medium plus NN as production medium) showed more enhanced root growth and TA production as compared with production media of White and NN medium and growth media of SH and 2B5 medium, respectively. However, bubble column bioreactor (BCB) cultures applying two-stage culture did not reveal the effective results despite of the each successful operation of two-stage culture in conical flasks and BCB cultures.

Key words : *Scopolia parviflora*, optimal culture medium, root line selection, tropane alkaloids, two-stage culture

INTRODUCTION

Plant useful metabolites have been used in many industries such as pharmaceutical products, cosmetics, food additives, and pigment. These metabolites are mostly supplied by direct extraction of plants. However, it can be a problem if the plants are in danger and endemic species. An alternative is employing *in vitro* culture system. Several products were found to be accumulated in cultured cells at a higher level than those in native plants, which are, for example, rosmarinic acid by *Collens blumei* (Ulbrich *et al.*, 1985) and ginsenosides by *Panax ginseng* (Ushiyama, 1991).

However, many reports have described that yields of desired products were very low in callus or suspension cultured cells. To elevate the productivity, several strategies have been established. Among them, two-stage culture can be an effective approach to increase the metabolites. Plant cell growth and metabolite production are generally shown to be negative correlation because of the required nutritional difference between primary and secondary metabolism (Sahai & Shuler, 1984). Mainly the production of these metabolites is not linked to growth, and therefore two-stage culture is necessary (Hoopen *et al.*, 2002). Based on these facts, growth medium and production medium can be defined as the best medium for growth and metabolite production, respectively. The first stage is operated in growth medium aiming to enhance cell biomass, and the second stage is carried out in production medium for elevated metabolite production.

Scopolia parviflora Nakai, a solanaceous perennial plant found in the deep mountain, is endemic to Korea and has been classified as being a rare endangered species (Ahn *et al.*, 1993). Since only *S. parviflora*

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produce tropane alkaloids (TA) among the Korean native plants, the supply of TA is entirely dependent on import in Korea which was provided by direct extraction of *Duboisia, Datura, Atropa* and *Hyoscyamus* species. In order to produce TA with higher productivity in Korean native plant, we have selected root lines which are SP72, the highest TA producing adventitious roots (Min *et al.*, in submitted). Thus, we carried out two-stage culture using SP72 of root line in both conical flasks and bubble column bioreactors (BCB) to investigate the productivity of TA in *S. parviflora.*

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 cultured as described in the report of Jung *et al.* (2002). On the basis of the result on root line selection, we determined SP72 of root line as the best on scopolamine production, and used the line in present experiments.

Test of culture media in conical flasks

For various experiments, the selected line was proliferated in the B5 (Gamborg *et al.*, 1968) liquid medium containing 5% (w/v) sucrose and 0.1 ㎎/ℓ IBA, which are determined in non-selected root line culture. The liquid cultures were maintained at 100 rpm and 25℃ under dark condition and subcultured every 4 weeks. In order to optimize the culture condition of SP72 root line, various culture media were conducted in adventitious root culture. The tested media were White (White, 1963), LP (Quoirin and Lepoivre, 1977), NN (Nitsch and Nitsch, 1969), WPM (Lloyd and McCown, 1981), MS (Murashige and Skoog, 1962), SH (Schenk and Hildebrandt, 1972), 1/4B5, 1/2B5, 1B5, 2B5 and 4B5. Each medium contained 3% (w/v) sucrose and 0.1 ㎎/ℓ IBA. The cultures were initiated by inoculating 0.5 g (F.W.) of roots and cultivated for 4 weeks.

Two-stage culture in conical flasks

On the basis of the results obtained from the optimal culture medium test, growth and production medium were selected for two-stage culture. Growth medium was defined as the best medium on the root growth, and production medium meant the best medium on the TA production. As the results of Fig. 1, growth medium was SH and 2B5 medium, and production medium was White and NN medium. The roots were initially cultured in growth medium for 10 days to increase biomass production. Thereafter, the growth medium was replaced with production medium, and the cultures were maintained for 5 days. The adventitious roots were cultivated in SH, 2B5, White, NN, and 1B5 medium and maintained for 15 days without replacement of culture medium for comparison with two-stage culture.

Two-stage culture in BCB cultures

By the result of two-stage culture in conical flasks, the combination of 2B5 medium as growth medium and NN medium as production medium showed the best result. Two-stage culture operating with 2B5 and NN medium was introduced into BCB cultures as the same methods in flask cultures. For comparison, one-stage culture was performed in BCBs operating with 1B5 medium containing 5% (w/v) sucrose and 0.1 ㎎/ℓ IBA which are optimal culture medium, carbon source, and growth regulator obtained in previous study. All of the bioreactors worked with 5 g (F.W.) of adventitious root inoculum at 0.4 vvm of air flow for 15 days as the results of Jung *et al.* (in submitted).

Measurement of root growth and TA contents

The adventitious roots were collectedand weighed as fresh weight. The root growth was represented by a growth index and TA contents were analyzed by HPLC and calculated using a calibration curve compared with standards and a co-chromatogram of the standards and samples as the protocol of Jung *et al.* (2002).

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. The statistical significance between

contrasting treatments was assessed by Duncan's multiple range test ($P = 0.05$).

RESULTS AND DISCUSSION

Determination of optimal culture medium for root growth and TA production in SP72 line of *S. parviflora* adventitious roots

For determination of optimal medium on the *S. parviflora* adventitious root growth and TA production, several culture media were tested for 4 weeks. Root growth was best in 2B5 medium and followed by SH, 1B5 and 1/2B5 medium (Fig. 1A). However, White, NN, 4B5 and 1/4B5 medium resulted in poor root growth. The production of hyoscyamine was more abundant than that of scopolamine in all tested media (Fig. 1B). The production of hyoscyamine was high in order of White, NN, WPM, 1/4B5 and 1/2B5 medium. In similar, scopolamine was produced with the maximum yield in White medium, whereas the minimum was obtained in 2B5 and SH medium. This disagreement between root growth and TA production can indicate that two-stage culture system can be one of the attractive strategies for increasing productivity of target compounds.

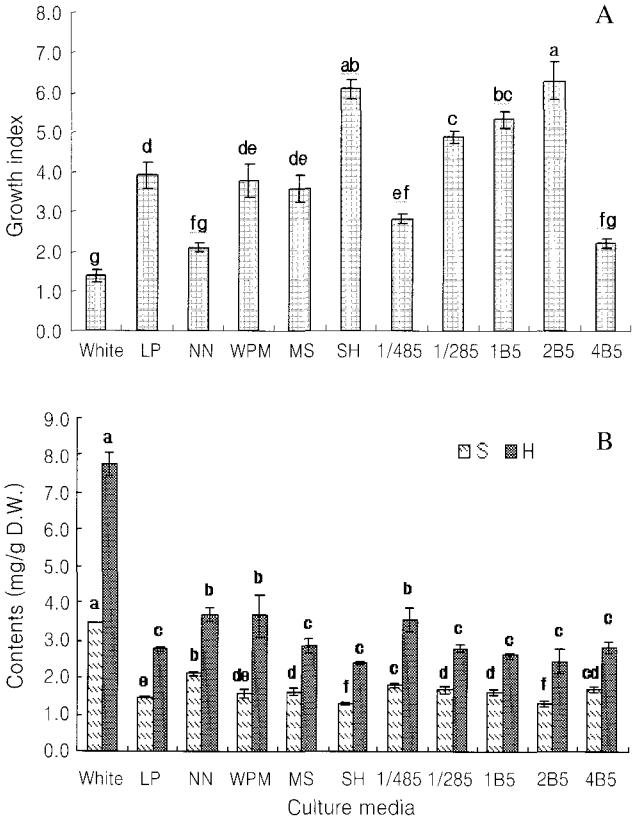

Fig. 1. Effects of culture media on growth (A) and TA production (B). 0.5 g (F.W.) of roots was cultured for 4 weeks in 100-mℓ flask containing 30 mℓ of various culture media. Each value represents the mean and standard deviation (S.D.) of three replicates (S: scopolamine, and H: hyoscyamine). Values bearing different letters in a line are significantly different at $P < 0.05$.

We previously reported the TA production on various culture medium of non-selected roots of *S. parviflora* when cultured in various culture media (Jung *et al.*, 2002). There is a little difference between SP72 root line and non-selected line in regard of optimal culture media, although both lines showed similar results. For instance, the non-selected root culture in 4B5 medium exhibited increased growth index whereas SP72 root was revealed poor growth. Hyoscyamine production was the maximum in non-selected and SP72 lines when cultured in 1/2B5 and White medium, respectively.

It is expected that adventitious roots of *S. parviflora* might require a lot of potassium and nitrogen source during growth period since SH and B5 medium consisted much of KNO_3 compared with other culture media, which showed higher levels of growth index. Furthermore, White and NN medium showing enhanced TA production are culture media containing low concentration of inorganic salts. Thus we suggest that *Scopolia* roots might demand great quantities of macro compounds for primary metabolism and require the least nutrients for secondary metabolism.

The effects of two-stage culture on the root growth and TA production in conical flask cultures

On the basis of the result of Fig. 1, 2B5 and SH medium were defined as growth medium, and White and NN medium were also defined as production medium. Two-stage culture was operated as 4 kinds of medium formulation. 2BW and 2BN meant that first stage was cultured in 2B5 medium as growth medium for 10 days and then transferred to second stage of White and NN medium for 5 days, respectively. SW and SN indicated that first stage was in SH medium as

growth medium and transferred to second stage of White and NN medium, respectively.

All of the two-stage cultures had enhanced growth and TA production compared to 1B5 medium (Fig. 2). The root growth was more increased in production medium of White and NN medium. TA production was also more increased of growth medium cultures of 2B5 and SH medium. Furthermore, the values of root growth and scopolamine production in two-stage cultures were higher than one stage culture with 1B5 medium, while the value of hyoscyamine production was not stimulated. The best result was obtained in 2BN, which enhanced yields on root growth and TA production compared to 1B5 medium. The result of Fig. 2 indicated that adventitious roots of *S. parviflora* required different nutrients for root growth and secondary metabolite production and the disagreement could be overcome by two-stage culture with 2B5 medium as growth medium and NN medium as production medium. Thus, the employing two-stage culture could provide increased TA production.

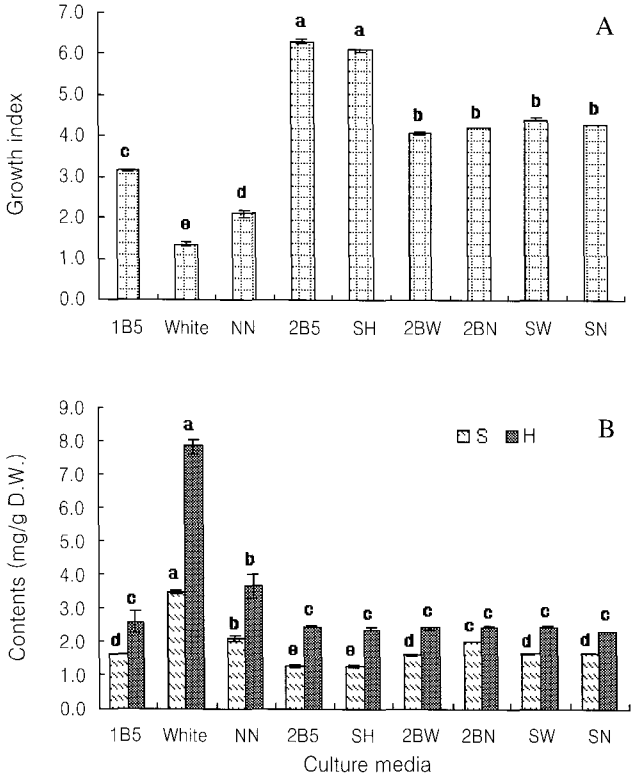

Fig. 2. Effects of two-stage cultures of *S. parviflora* adventitious roots. 2BW : 2B5+White medium, 2BN : 2B5+NN medium, SW : SH+White medium, and SN : SH+NN medium (S : scopolamine and H : hyoscyamine). Values bearing different letters in a line are significantly different at $P < 0.05$.

Zhang *et al.* (1997) also carried out two-stage culture in regard to optimal culture temperature in strawberry cell culture. They found that maximum growth occurred at 30℃. However, the lower temperature induced an increased level of anthocyanin content and as a result, the maximum anthocyanin production was obtained at 20℃. The application of two-stage culture increased anthocyanin production 1.8 and 4-fold over that of cultures at 20 and 30℃, respectively. Tom *et al.* (1991) reported that in *Catharanthus roseus* suspension culture, total indole alkaloid production was 10 times higher for two-stage culture than for one-stage cultures. Therefore, if the optimal culture conditions for cell growth and metabolite production are determined, two-stage culture system through formulating the two conditions may provide positive effects in different species and tissue cultures.

The effects of two-stage culture on the root growth and TA production in BCB cultures

In conical flask cultures, two-stage culture enhanced both root growth and TA production as compared with control of 1B5 medium (Fig. 2). However, two-stage culture did not revealed the effective result in bioreactor cultures (Fig. 3). Only root growth was slightly increased in two-stage culture, but there was no significantly difference as a result of statistical analysis, and both scopolamine and hyoscyamine contents were decreased as compared with one-stage culture.

In red pigment production by *Carthamus tinctoriuscells*, growth levels of cells in an Erlenmeyer flask and a seesaw type bioreactor were higher than those in a stirred tank bioreactor and a BCB for the first stage, whereas the reversed results were found in pigment formation for the second stage (Hanagata & Karube, 1994). They supposed that the results were caused by the high initial volumetric oxygen transfer coefficient (kL) in a stirred tank bioreactor and a BCB. On the other hand, many researchers have reported that after transferring the cell culture to bioreactor, a significantly decreased alkaloid production

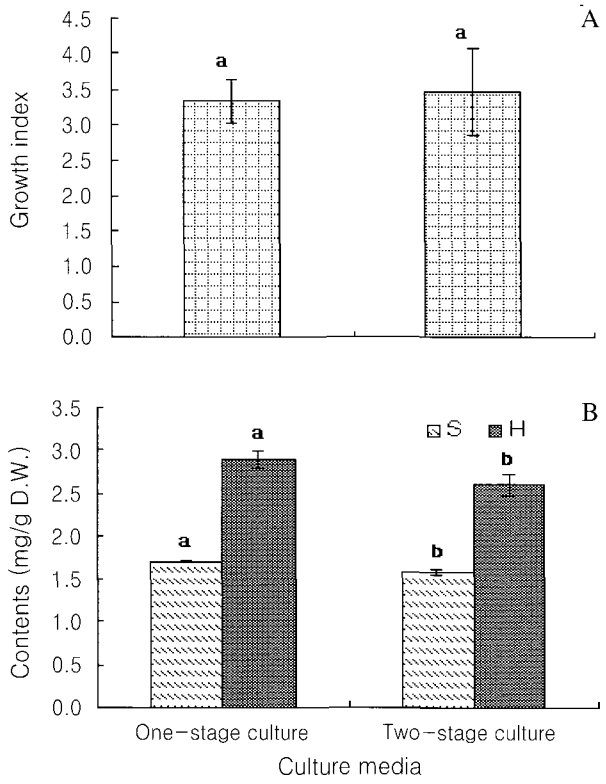

Fig. 3. The effect of two-stage culture on root growth and TA production. Two-stage culture was operated in 2B5 and PM as GM and PM, respectively. The error bars indicate standard deviation of three independent experiments (S : scopolamine, and H : hyoscyamine). Values bearing different letters in a line are significantly different at $P < 0.05$.

is often observed (Zhao *et al.*, 2000; Schiel & Berlin, 1987; Kargi & Rosenbergm, 1987). And they expected that it may be due to improper culture conditions, such as nutrient supply, gas factor and other unknown reasons in bioreactors.

In our previous study with a BCB, the bioreactor culture in 1B5 medium supplemented with 5% (w/v) sucrose and 0.1 ㎎/ℓ IBA showed positive effects on root growth and TA production as compared with a conical flask culture (Jung *et al.*, in submitted). However, scale-up culture applying two-stage culture was unsuccessful (Fig. 3). In particular, both scopolamine and hyoscyamine production were decreased in a BCB introducing two-stage culture. On the contrary, we found that two-stage culture formulating 2B5 and NN medium in a conical flask and scale-up culture in optimized conditions such as culture medium, carbon source, growth regulator, inoculum density, aeration level, and culture period were successfully achieved. On the basis of these incongruous results, we expected that difficulty in manipulation of scale-up culture such as replacement of growth medium with production medium and several unknown factors might influence the productivity. And thus the problem might be solved by designing bioreactors with automatic medium transfer system in order to shorten medium exchange time and further exclude the environmental unknown factors.

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