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Production of tropane alkaloids by small-scale bubble column bioreactor cultures of *Scopolia parviflora* adventitious roots

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Abstract

The mass production of tropane alkaloids from adventitious root cultures of *Scopolia parviflora*, in small-scale bubble column bioreactor (BCB) was attempted. Adventitious roots of *S. parviflora* produced relatively enhanced levels of scopolamine and hyoscyamine in bioreactor compared to flask type cultures, and rapidly produced root clumps, with continuously increasing biomass throughout the culture period. The production of scopolamine and hyoscyamine in the top and bottom regions of root clumps were higher than in the core region. The adventitious root cultures of *S. parviflora* in the BCB required a relatively high level of aeration. The optimized conditions for the bioreactor culture growth and alkaloid production were found to be 3 g of inoculum, on a fresh weight basis, a 15-day culture period and 0.4 vvm of airflow. The elicitation by *Staphylococus aureus* increased the specific compound of scopolamine, while the production of hyoscyamine was slightly inhibited in BCB cultures.

Keywords: Aeration; Bubble column bioreactor; Inoculum density; *Scopolia parviflora*; Tropane alkaloids

1. Introduction

Higher plants produce diverse ranges of pharmaceutically important secondary metabolites (Cho et al., 2003). *Scopolia parviflora*, a solanaceous perennial plant, is endemic to Korea and recently classified as rare endangered species. *S. parviflora* produces considerable levels of tropane alkaloids with anticholinergic activity (Baiza et al., 1998). Bioreactor culture studies have been initiated in solanaceous crops, such as *Atropa, Datura, Duboisia* and *Hyoscyamus* species for the production of secondary metabolites. However studies on use bioreactors for the commercial production of scopolamine and hyoscyamine from *S. parviflora* are limited.

Cultures of adventitious or hairy roots are potential source for the production of valuable plant secondary metabolites on commercial scale. Biosynthesis of scopolamine and hyoscyamine is correlated with root differentiation (Endo and Yamada, 1985). Dedifferentiated cell culture studies for the production of scopolamine and hyoscyamine were found unsuccessful, as they are predominantly synthesized in roots. This suggests that development of suitable methods for large-scale culturing of adventitious and/or hairy roots would be a viable approach for the production of tropane alkaloids. The ability of hairy roots to grow to high density and to produce significant amount of secondary metabolites makes them a suitable system for large-scale culture in reactor (Wilson, 1997). Keeping this in view, we devised bubble column bioreactor (BCB) to scale-up of culturing of adventitious roots. Many factors are severely affected during scale up

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of any kind of cell culture. These include oxygen transfer rate, heat transfer, mixing and the associated shear stress, superficial air velocity and culture age and stability (Humphery, 1998; Kim et al., 2002). Scale up of hairy root cultures remains very challenging, and despite recent advances, the objectives of optimal growth and production in bioreactors are far from being reached (Kim et al., 2002; Ogbonna et al., 2001; Kaewpingtong et al., submitted). In the present study, the optimal conditions, such as culture period, initial inoculum density and aeration, required for culturing adventitious or hairy root cultures of *S. parviflora* in a BCB for the production of scopolamine and hyoscyamine were evaluated.

2. Methods

2.1. Plant material and adventitious root culture

The mother plant of *S. parviflora* was provided by the National Arboretum of Korea. The adventitious roots were induced from the rhizome of *S. parviflora* (Jung et al., 2002) and maintained by sub culturing in B5 medium supplemented 50 g l^{-1} sucrose and 0.1 mg l^{-1} IBA from last three years. The media used in flask cultures, consisted of B5 medium supplemented with 50 g l^{-1} sucrose and 0.1 mg l^{-1} IBA. The liquid culture was established by the inoculation of 0.5 g fresh weight (F.W.) of roots into a 100 ml conical flask containing 30 ml medium. The flasks were maintained in the dark at 25 ± 1 °C, 100 rpm on a rotary shaking incubator.

2.2. Preparation of the bubble column bioreactor (BCB) culture

The bioreactor consisted of a glass vessel culture chamber (7.5 × 7.5 × 25 cm), air inlet and outlet, sampling ports, accessory connector and an air filter (0.20 μM pore size, Midisart® 2000, Sartorius, Germany), connected with silicon tubes. The sampling ports and accessory connector were locked for efficient operation. The internal diameter of the bioreactor was 6.5 cm, with a working volume of 300 ml (Fig. 1). The pH of medium used in the bioreactor was adjusted to 5.8 with 0.1 N NaOH or HCl by pH controller before autoclaving at 121 °C for 15 min. Filter-sterilized air was supplied through a sparger from the bottom of the bioreactor. Five grams F.W. of 14-day-old randomly cut 1.5–2 cm segments of adventitious roots was used as inoculum. The entire operation was carried out at 25 ± 1 °C in the dark.

2.3. Optimization of culture condition

A time-course test for the bioreactor was accomplished with 5 g of inoculum density fresh roots, operating with a 0.3 vvm air flow, for 25 days, with samples taken at 5 day intervals. To determine the optimal inoculum density, different levels of inoculants (3, 5, 10, 15 and 20 g F.W.) were used separate experiments. Efficient aeration is an important factor in bioreactor culture studies. Therefore, air flows of 0.1, 0.2, 0.3, 0.4 and 0.5 vvm were evaluated with the 5 g (F.W.) inoculum density.

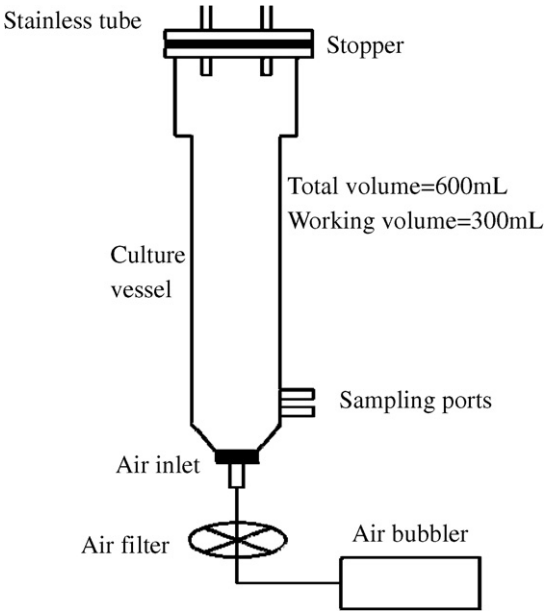

Fig. 1. Design of the bubble column bioreactor (BCB) used in this study. The vessel volume was 600 ml (7.5 × 7.5 × 25 cm) and the working volume was 300 ml. The glass growth chamber was connected to an air bubble to provide oxygen. Supplied oxygen was sterilized by passing an air filter and bubble were created through a sparger.

2.4. Measurement of root growth, conductivity, and dissolved oxygen

The growth of adventitious roots was measured as described in Jung et al. (2002). The adventitious roots were separated from the medium, blotted and weighed. The growth index (G.I.) was equal to the fresh weight of harvested roots minus fresh weight inoculated roots divided by the fresh weight inoculated roots. The nutrient, as a measure of conductivity, and dissolved oxygen (DO) utilization by the growing roots was recorded for a period of 25 days. The electrical conductivity and DO of the medium were measured using an electrical conductivity electrode (MultiLine P4, Tetracon® 325) and DO electrode (MultiLine P4, CellO × 325), respectively.

2.5. Extraction and quantification of scopolamine and hyoscyamine

Tropane alkaloids from harvested roots and media were prepared for HPLC analysis according to the method of Kang et al. (2004). The HPLC separated scopolamine and hyoscyamine were analyzed using EI mass spectrometer (JMS-AX505 WA).

2.6. Application of effective elicitor

Based on previous studies, 0.13 ml ml^{-1} of elicitor was added to 18-day-old BCB cultures containing B5 medium containing 50 g l^{-1} sucrose and 0.1 mg l^{-1} IBA, and further grown for 72 h, before harvested and evaluated for root growth and tropane alkaloid content.

2.7. Statistical analysis

The results were expressed as the average of three separate experiments. The error bars indicate the standard deviation (SD) from the mean of each replicates. The statistical significance between contrasting treatments was assessed by Duncan's multiple range test ($p = 0.05$).

3. Results and discussion

3.1. Kinetics of adventitious roots in a bioreactor culture

The biomass reached 7.2 times higher than that of the inoculum used. The volume of culture medium decreased to almost half of the original volume by the end of the growth period (Fig. 2a). The growth pattern of adventitious roots in the bioreactor was almost on par with the growth rate in flask cultures. However, in BCB cultures, root growth in late growth phase was enhanced compared with flask cultures. Jung et al. (2003) reported that growth pattern of adventitious roots in flask culture was showed lag phase (0–4 days of culture), exponential phase (4–24 days of cultures), stationary phase (24–30 days of culture) for 30 days of cultures.

During the early stage of growth, both conductivity and DO concentration decreased drastically, despite of continuous supply of air at 0.3 vvm (Fig. 2b). These results suggest that adventitious roots require high amount of nutrients and oxygen for their growth. The production of hyoscyamine was approximately twice that of scopolamine (Fig. 2c). The production of scopolamine and hyoscyamine were fairly constant throughout the growth, on dry weight basis, and correlated with increase in growth of adventitious roots. However, scopolamine and hyoscyamine content in culture medium was low (less than 1 mg l^{-1}) without elicitation.

Culturing of adventitious and hairy roots of *Atropa, Datura, Duboisia* and *Hyoscyamus* have been reported for the production of scopolamine and hyoscyamine. However, reduction in the alkaloid contents was observed during scaling up process (Kwok and Doran, 1995). The present study indicate that the adventitious roots of *S. parviflora* produced enhanced levels (3–4 folds) of scopolamine and hyoscyamine in the BCB compared to flask cultures (Jung et al., 2003), without much change in the growth parameters. Therefore, the use of adventitious roots of *S. parviflora* for the production of scopolamine and hyoscyamine in bioreactors would seem to be promising.

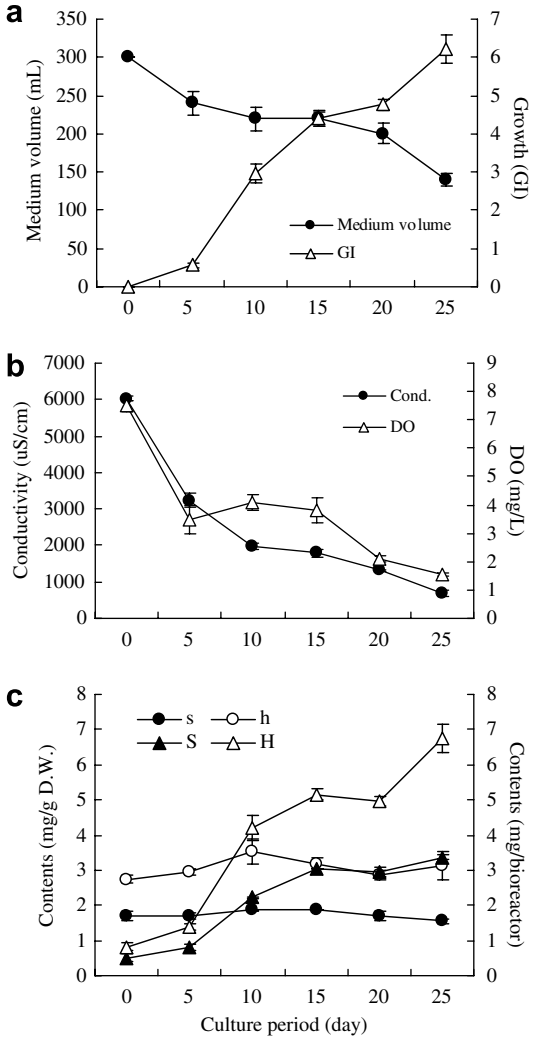

Fig. 2. Time-course studies on adventitious roots growth of *S. parviflora* in a small-scale BCB. (a) the consumption of cultured medium in relation to the root growth, (b) the conductivity (Cond.) and DO concentration (DO), and (c) the production of scopolamine (s, S) and hyoscyamine (h, H). Small letters (s and h) indicate the contents per g of dry weight and the capital letters (S and H) indicate contents in total biomass.

3.2. The effects of different inoculum densities

One of the factors that determine the productivity in *in vitro* cultures is the optimal inoculum density (Lee and Shuler, 2000). Maximum biomass was obtained when 15 g (F.W.) of adventitious roots were fed into the bioreactor, but optimal inoculum density was obtained when 3 g (F.W.) of roots were inoculated into the bioreactor (Fig. 3). High inoculum densities resulted in poor growth, as the adventitious roots rapidly reached their stationary phase, which limits the availability of nutrients and oxygen and affect the production of secondary metabolites.

The adventitious roots in BCB became tangled and formed root ball like structures. The growth pattern of the adventitious roots were categorized into (1) the actively growing region, including the top and bottom regions of the root ball, the young growing roots on the periphery

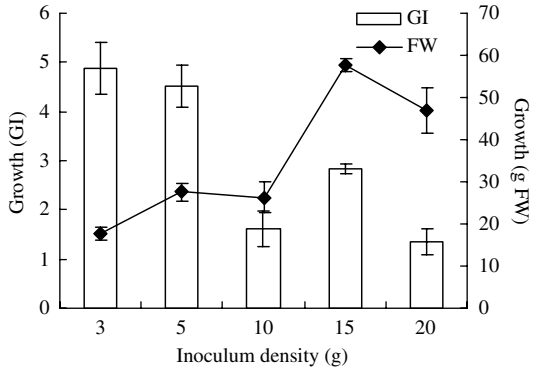

Fig. 3. Effect of inoculum densities on the growth of adventitious roots in BCB. The results are presented as both the fresh weight (F.W.) and growth index (GI). The cultures were cultivated for 3 weeks under dark conditions.

that appeared yellowish white in color, which branched outward, and (2) the suppressed growing region, the inside of the root, which turned brownish yellow in color, with old and senescent tissues (data not shown). On the basis of these morphological differences in the root ball, the productions of scopolamine and hyoscyamine were analyzed in the different regions. The outside of the root ball was defined as the top and bottom regions that were exposed to sufficient nutrients and oxygen. The inside of root the ball was defined as the core region that had a restricted supply of nutrients and oxygen.

The production of scopolamine increased with increasing inoculum density, while that of the hyoscyamine was negatively correlated (Table 1). The range of scopolamine contents was 1.44–1.82 g g^{-1}dry weight basis and hyoscyamine contents were 2.66–3.29 g g^{-1} dry weight. The maximum scopolamine content was obtained from inside of BCB with 15 g of inoculum density, while hyoscyamine content has minimum at this condition. In this study, hyoscyamine content was approximately 2-fold more than that of scopolamine. Scopolamine is epoxidation product of 6β-hydroxyhyoscyamine (Hashimoto et al., 1989), it is expected that high level of scopolamine synthesis should occur in outside region. However, our results indicate that the regional specific production of hyoscyamine was distinct but not scopolamine, the levels of which remained same in outside and inside regions of adventitious roots (Table 1). However increase in the inoculum density of *S. parviflora*, resulted in increased production of scopolamine, whereas the production of hyoscyamine was reduced.

Table 1
Effect of inoculum densities on the production of scopolamine and hyoscyamine in BCB

Inoculum density (g F.W.)	Scopolamine		Hyoscyamine	
	Outside*	Inside	Outside	Inside
3	$1.48 \pm 0.00^{f**}$	1.49 ± 0.04^{f}	3.29 ± 0.14^{b}	3.11 ± 0.08^{cbd}
5	1.55 ± 0.03^{e}	1.44 ± 0.02^{f}	3.20 ± 0.17^{cb}	2.66 ± 0.09^{e}
10	1.69 ± 0.04^{dc}	1.65 ± 0.00^{d}	3.66 ± 0.27^{a}	3.11 ± 0.07^{cbd}
15	1.71 ± 0.04^{dc}	1.81 ± 0.01^{a}	2.98 ± 0.13^{cd}	2.88 ± 0.23^{ed}
20	1.79 ± 0.01^{ab}	1.75 ± 0.07^{bc}	3.14 ± 0.05^{cbd}	3.16 ± 0.16^{cbd}

Values bearing different letters in a column are significantly different at $p < 0.05$.

* 'Outside' was defined as the top and bottom regions of a root ball, and 'Inside' as the core region within a 2 cm diameter of a root ball.

** Contents are expressed as mg g^{-1} dry weight (D.W.), with the standard deviation (SD).

3.3. The effects of different air flows

The factor that influence effective oxygen transfer in plant cell cultures must be carefully analyzed for the bioreactor designed (Chattopadhyay et al., 2002). The present study revealed that aeration (0.1–0.5 vvm) significantly affects the growth of adventitious roots and production of alkaloids. Hardly any aeration occurred at 0.1 vvm, with the culture medium remaining stagnant and no formation of visible bubbles. The root ball grew under absolutely submerged conditions. However, when the bioreactor was operated at 0.5 vvm, turbulent flow was produced, and foam covered the root ball. The root ball floated due to the formation of bubbles, and grew better (data not shown). The GI values were augmented by an increase in the aeration from 0.1 to 0.4 vvm (Fig. 4a). Poor aeration,

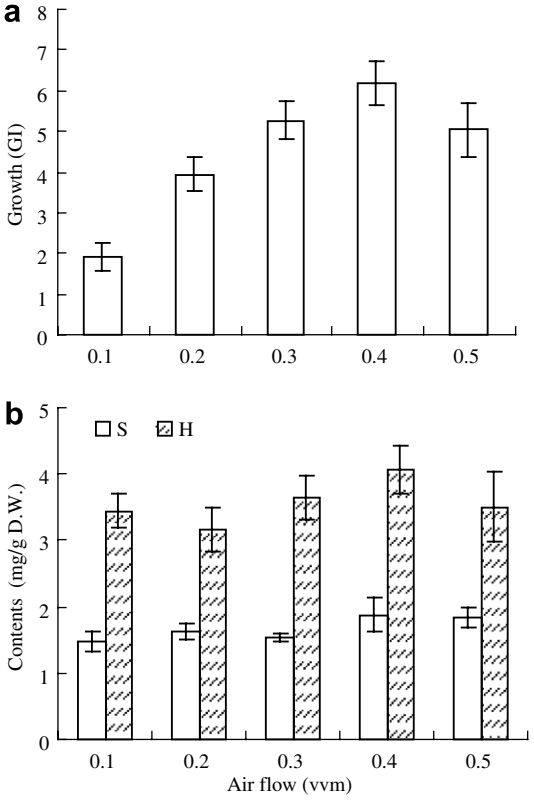

Fig. 4. Effects of aeration rates on the root growth (a) and production of scopolamine and hyoscyamine (b) in BCB. The error bars indicate the standard deviation (SD) of three independent experiments. (S: scopolamine, and H: hyoscyamine).

0.1 vvm, resulted in stunted root growth, roots becoming gray in color and with no formation of tangled root ball. The most effective aeration for root growth was achieved with 0.4 vvm.

The effective productions of scopolamine and hyoscyamine, as well as enhanced root growth, were achieved at 0.4 vvm (Fig. 4a and b). Although the different inoculum densities affected the root growth, and scopolamine and hyoscyamine production, these were more dependent on the aeration than the inoculum density in the bioreactor. In spite of poor growth and cell death at 0.1 vvm aeration, the scopolamine and hyoscyamine productions were on a par with the rest. In general, plant cells require less oxygen, ranging from 0.05 to 0.4 vvm, than microorganisms as they have large vacuoles, and a slow growth and metabolism. Our studies indicate that the adventitious roots of *S. parviflora* require high concentration of oxygen compared to other plant cells and roots cultures. The results also support that scopolamine biosynthesis is oxygen-dependent, requires molecular oxygen to activate one of the key enzymes, H6H (Hashimoto et al., 1989) and the production of hyoscyamine is controlled by available dissolved oxygen in the media (Fig. 4b). Excessive aeration, at 0.5 vvm, was detrimental to root growth and for the productions of scopolamine and hyoscyamine. This could be due to either 'stripping off' of key volatiles, such as carbon dioxide and ethylene, from the cultured medium, or a direct oxygen toxicity resulting from a high level of DO (Schlatmann et al., 1993). The low aeration rates affected the root growth rates, despite the maintenance of a high DO concentration.

3.4. The effects of elicitor

The inhibition of root growth and production of metabolites by the bacterial elicitor was less known in BCB cultures. The adventitious roots vigorously grew throughout 72 h and cell death happened at 144 h of co-culture with bacterial elicitor. The elicitator from *S. aureus* increased the production of scopolamine, while the production of hyoscyamine was slightly inhibited (data not shown). The scopolamine content increased up to 72 h and reached the maximum yield of 25.7 mg g^{-1} on dry weight (D.W.) basis, which is 9.9 times higher than control.

Many studies have reported significant reduction in alkaloid production after transferring the cell culture to bioreactor. It was attributed to improper culture conditions, such as nutrient supply, gas factor and others unknown in bioreactor studies, (Zhao et al., 2000; Schiel and Berlin, 1987; Kargi and Rosenbergm, 1987) a major drawback in the scaling up process. The results of the present study provides useful information on the conditions, such as a 15-day culture period, a 3 g (F.W.) inoculum density and an air flow of 0.4 vvm required for optimal increase in biomass, scopolamine and hyoscyamine production from the adventitious roots in the BCB, and elicitation.

4. Conclusions

A small-scale bioreactor for growing adventitious roots of *S. parviflora* was successfully developed in BCB. The productivity of scopolamine and hyoscyamine were found dependent on inoculum density and air flow. These findings suggest that *S. parviflora* adventitious roots require a relatively high concentration of oxygen required and tropane alkaloid biosynthesis is regulated by the DO to certain extent. However scaling-up of adventitious roots culture still remains very challenging despite recent advances. Continuous attempts are necessary to develop protocols for optimal growth root cultures, and scopolamine and hyoscyamine production in bioreactors. The results of our study contribute some crucial information for optimization and development of bioreactor technology for adventitious root cultures of *S. parviflora* for the production of tropane alkaloid.

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