Increase of Scopolamine Production by High Density Culture of Duboisia myoporoides Roots

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A circulation culture system was established for the high density root culture of Duboisia roots. It consists of a vessel for root culture, an aeration tube, a medium reservoir, and a peristaltic pump. Medium saturated with pure oxygen was circulated continuously through the culture vessel and the medium reservoir, and was pumped back into the aeration tube by the pump. Duboisia roots could be cultured at densities of up to 120 g dry weight (DW) dm⁻³ with no decrease in scopolamine content, this density being about 20 times the amount that can be used in ordinary flask culture. The final scopolamine yield at the end of 3 weeks was 1350 mg dm⁻³.

The production of secondary metabolites in cultured plant cells has been well studied; but few commercial applications have been made because of technological problems and the high processing costs of industrial scale production. Moreover, metabolites such as tropane alkaloids mainly are produced in differentiated roots, and root culture is more difficult than undifferentiated cell culture. Cultured roots are easily damaged by shear stress or impeller rotation when a stirred-tank reactor is used, and although bioreactor systems suitable for root culture have been proposed, their productivities are low.

High density culture is one way to enhance the productivity per culture tank. The high density plant cell culture methods developed so far have been reviewed by Tanaka (1987). The main problem with these methods is that an increase in cell density causes a decrease in the volumetric oxygen transfer coefficient (k_o,a) because of insufficient mixing and gas dispersion. These phenomena are more pronounced in airlift than in stirred bioreactors.

We have established a circulation culture system that is suitable for root culture at high density and report here its use for the production of scopolamine with roots of Duboisia myoporoides.

Materials and Methods

Root culture. Roots of Duboisia myoporoides R. Br., line RB-1, induced hormonally, were subcultured in Nitsch and Nitsch (NN) liquid medium every 3 weeks. About 0.1 g fresh weight (FW) of roots was inoculated into a 100-mL Erlenmeyer flask containing 20 mL of NN medium with 3% sucrose and 10 μM indolebutyric acid (IBA), then the flask was incubated at 25°C in the dark on a rotary shaker at 100 rpm (agitation diameter 25 mm).

Circulation culture system. The circulation culture system established for the high density culture of RB-1 roots consisted of a vessel (20-40 mL) for root culture, an aeration tube (30 or 100 mL), a reservoir for the medium (0-600 mL), and a peristaltic pump (Fig. 1). The vessel, the aeration tube, and the pump were connected by silicon and glass tubing (internal diameter, 4 mm). The inlet and the outlet of the culture vessel were each covered with a stainless steel filter (80 mesh) that prevented the roots from flowing out. Medium saturated with pure oxygen was circulated continuously through the culture vessel and the medium reservoir, and pumped back into the aeration tube by the pump.

Results and Discussion

Establishment of the circulation culture system

To establish a method for the high density culture of Duboisia roots, we investigated the efficiency of various bioreactors on the basis of the following criteria: (1) a sufficient and uniform supply of oxygen; (2) an adequate supply of nutrients for the inoculum size; (3) avoidance of damage to the roots by shear stress or impeller rotation.

Airlift bioreactors were proved not appropriate for high density culture because of insufficient mixing and gas dispersion. Stirred tank bioreactors are appropriate for high density cell suspension culture but not for root
culture, because roots are sensitive to physical stress.\(^6\)

We therefore devised the circulation culture system shown in Fig. 1. It consists of a vessel for root culture, an aeration tube, a medium reservoir, and a peristaltic pump, all connected by silicon and glass tubing. By separating the culture vessel from the aeration tube, we could supply sufficient oxygen for growth without causing damage to the roots.

Effects of vessel shape on root growth and scopalamine production

The ratio of the length (L) to the inside diameter (D) (L/D ratio) of a cylinder-shaped culture vessel in the circulation system was investigated for the root culture of *D. myoporoides* R. Br., line RB-1. This system consisted of a 20-ml culture vessel, a 100-ml aeration tube, an 80-ml medium reservoir, and a peristaltic pump that provided a flow rate of 10 ml/min. Two grams FW (2.5 g DW dm\(^{-3}\)) of RB-1 roots was inoculated into 200 ml of NN medium with 5% sucrose and 10 \(\mu\)M IBA, then cultured for 3 weeks.

The final amount of root growth and scopalamine productivity are shown in Fig. 2. Root growth was 64 g DW 100 ml \(^{-1}\) at a L/D ratio 0.5, and the scopalamine content was 1.3% at a L/D ratio 1.0. At excessive ratios (L/D: 0.05 and 20), non-uniform root clusters were formed in the vessel and the supply of oxygen and nutrients to the inner parts of the clusters was disturbed, resulting in a decrease both in root growth and scopalamine yield. As the scopalamine yield was highest (760 mg dm\(^{-2}\)) when the L/D ratio was about 0.5, vessels with this L/D ratio were used in the subsequent experiments.

Effects of the dissolved oxygen concentration and \(k_{4Ia}\) on root growth and scopalamine production

RB-1 roots were cultured in this new circulation system to examine the effects of the dissolved oxygen (DO) concentration on root growth and scopalamine production. The system consisted of a 40-ml culture vessel, a 100-ml aeration tube, a 60-ml medium reservoir, and a peristaltic pump that provided a flow rate of 20 ml/min. Two grams FW (1.25 g DW dm\(^{-3}\)) of RB-1 roots was inoculated to 200 ml of NN medium and cultured for 3 weeks. Nitrogen and oxygen were mixed, the mixture being adjusted to the corresponding oxygen concentrations (Fig. 3). Values indicate the oxygen concentration at the inlet of the culture vessel, the point at which the aerated medium enters.

Up to a concentration of 40 ppm oxygen, both root growth and the scopalamine content increased. At 40 ppm oxygen, root growth was 28 g DW dm\(^{-3}\), and the scopalamine content was 1.3%, 1.4-fold the values at 8 ppm oxygen. As a result, the scopalamine yield at 40 ppm was twice that at 8 ppm, the concentration usually used in ordinary flask culture, but when the oxygen concentration was increased to 50 ppm, the scopalamine content decreased, because the pressure in the system rose to 1.25 atm.

As medium saturated with pure oxygen is pumped to the culture vessel by a peristaltic pump at a flow rate of 1/2 the volume of the culture vessel per minute, the \(k_{4Ia}\) is estimated to be 30 h\(^{-1}\). The effects of the apparent \(k_{4Ia}\) on root growth and scopalamine production were also examined by varying the flow rate from 1/4 to 2 times the volume of the vessel per minute. As none of the variations markedly stimulated or inhibited root growth or scopalamine production by the root cultures (data not shown), the flow rate of 1/2 the volume of the vessel per minute was used in the subsequent experiments.
Effects of inoculum size on root growth and scopolamine production

Various amounts of RB-1 roots were inoculated into the culture vessels, and the effects of inoculum size on root growth and scopolamine production were investigated. The circulation system consisted of a 30-ml culture vessel, a 100-ml aeration tube, a medium reservoir, and a peristaltic pump that operated at a flow rate of 15 ml/min. In this experiment, the volume of the medium was varied in proportion to the inoculum size. Basically, 100 ml of NN medium was used per 1 g FW of roots.

The values for final root growth and scopolamine production are shown in Fig. 4. The relation between inoculum size and final root growth for inocula of less than 5.0 g DW dm\(^{-3}\) was approximately linear. Although root growth also increased at more than 5.0 g DW dm\(^{-3}\) of inoculum, the rate (shown by the slope of the line in Fig. 4) was markedly lower. At 5.0 g DW dm\(^{-3}\) of inoculum, final root growth at the end of 3 weeks was 120 g DW dm\(^{-3}\), and the scopolamine yield was 1350 mg dm\(^{-3}\). Root density was about 20 times the amount obtained by the usual flask culture method used for subculture (data not shown). High scopolamine production was maintained with inocula of less than 5.0 g DW dm\(^{-3}\), but an inoculum of more than this inhibited scopolamine production.

As RB-1 roots grew in the circulation system, they filled the vessel, forming vessel-shaped clusters. The addition of auxin to the medium used for root culture induces abundant lateral root primordia and subsequent lateral root elongation.\(^{1,3}\) In the culture vessel, RB-1 roots can elongate to areas where oxygen and nutrients are sufficient; therefore, they appear to grow uniformly at high density.

Reduction of the volume of the medium

In the inoculum-size experiment, the volume of the medium was varied in proportion to the amount of inoculum. Optimum conditions consisted of a circulation system made up of a 30-ml culture vessel, a 100-ml aeration tube, a 470-ml medium reservoir, and a peristaltic pump that provided a flow rate of 15 ml/min. Six grams FW (5 g DW dm\(^{-3}\)) of RB-1 roots was inoculated into the 600 ml of medium necessary for culture with this size culture vessel. One way to reduce the volume of medium needed is to keep the concentrations of its components equal to those of normal culture and to eliminate substances which have adverse effects on culture.\(^{14}\) Therefore, the fed-batch and fed-batch plus perfusion methods were investigated.

Table I gives the conditions. To start high density culture, modified NN medium was used, to which 2.5 mm KH\(_2\)PO\(_4\), 100 \(\mu\)m IBA, and 5% sucrose had been added. The media for the fed-batch and perfusion experiments are also given in this table. The concentrations of the components of the medium for the fed-batch culture were calculated from the amount of each NN component incorporated in roots grown at the usual density used for ordinary batch culture. IBA was added to the medium in proportion to the inoculum density at the beginning of culture. Because phosphate and ammonium are incorporated into roots within 7 days during ordinary batch culture, these nutrients were added only on each of the first seven days of culture. Other nutrients were added gradually to the fed-batch culture of RB-1 roots so that the concentrations of the nutrients in the medium would not become too high during the initial stage of culture.

The circulation system consisted of a 30-ml culture vessel, a 30-ml aeration tube, and the peristaltic pump that circulated the medium at a flow rate of 15 ml/min, but no
medium reservoir. In this experiment, the volume of the aeration tube was 30 ml. Six grams FW (5.0 g DW dm\(^{-2}\)) of RB-1 roots was inoculated into 60 ml of modified NN medium, and cultured in the system for 3 weeks. The conditions for the fed-batch or fed-batch plus perfusion cultures are given in Table I.

A reduction in the volume of the medium was possible with the combination fed-batch plus perfusion B, but not with perfusion A or fed-batch culture alone. Perfusion A had adverse effects on scopalamine production. The initial volume of the culture medium could be reduced from 600 to 60 ml, as shown in Tables I and II.

Matsubara et al.\(^{14}\) reported that *Coptis japonica* cells were cultured at densities of up to 75 g dm\(^{-3}\) in a culture tank fitted with a hollow-paddle type stirrer. Their culture system, however, was not appropriate for root culture because roots were easily damaged by shear stress. Kondo et al.\(^{7}\) compared three types of bioreactors for their suitability for growing hairy roots of *Daucus carota*. They found a turbine-blade reactor (a stirred tank where the stirring compartment was separated from the cell compartment) and a rotating drum reactor with immobilized hairy roots gave the best growth rate, and the root density of 16 g dm\(^{-3}\) was achieved in 48 days. Taya et al. also compared the growth of hairy roots in bioreactors and they found the air reactor with cells immobilized on a polyurethane foam sheet to be superior to Erlenmeyer flasks, stirred tank reactors, airlift reactors, and airlift reactors where the medium was circulated along the roots, leaving the roots exposed to air for most of the culture time in the free root airlift reactor. A root density of 11 g dm\(^{-3}\) was achieved in 31 days. Hilton and Rhodes\(^{5}\) reported that hairy roots of *Datura stramonium* were cultured at densities of up to 21 g dm\(^{-3}\) after 37 days in a modified stirred tank reactor. In this study, *Dauoisia* roots were cultured at densities of up to 120 g dm\(^{-3}\) after 21 days with no decrease in scopalamine content in the circulation culture system. This density is the highest that has been reported.

Our circulation culture system proved very useful for obtaining high-density root cultures. Although the experiments were done on a small scale, with certain modifications, the system should be applicable to the large scale culture of *Dauoisia myoporoides* roots as well as to the culture of other species for biotransformation studies or the production of useful compounds mainly produced in the roots.

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References