Tropane Alkaloid Production in Root Cultures of *Duboisia myoporoides* Obtained by Repeated Selection

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Repeated selection of root lines that were highly productive for scopolamine was investigated for *Agrobacterium rhizogenes*‐transformed and untransformed roots of *Duboisia myoporoides*. Lines highly productive for scopolamine could be selected by repeated selection of transformed roots, but not by repeated selection of untransformed ones. In transformed root cultures, the scopolamine content of the highest scopolamine‐producing root line after 8 selections was 3.2% dry weight (DW) as compared to 0.15% DW for the parent line. The total alkaloid contents and scopolamine/hyoscyamine ratio also increased. Repeated selection decreased growth from 7.0 to 2.2 g/liter, but there was no correlation between growth and scopolamine content in the selected transformed root cultures. This enhancement of scopolamine content and decrease of growth appears to be caused by the heterogeneous nature of the transformed roots.

The tropane alkaloids hyoscyamine and scopolamine are used medicinally as spasmolytics and anesthetics. These alkaloids are mainly synthesized in the roots then translocated to aerial plant parts.¹ Studies of the production of tropane alkaloids by cultured cells and root tissues of various alkaloid‐containing Solanaceae² ⁻⁶ show that differentiation of root tissue is necessary for alkaloid production. Root cultures can be established by the manipulation of the auxin levels in the culture medium or by the transformation of plants with *Agrobacterium rhizogenes*. *A. rhizogenes* induces hairy root diseases in dicotyledonous plants⁷ because of the transformation of plant cells due to the introduction of the T‐DNA of the Ri plasmid into plant genomes through a process similar to that which operates in the crown gall diseases produced by *A. tumefaciens*.⁸ Transformed roots can be cultured in liquid media without growth regulators, and such cultures have been proposed as suitable for the production of tropane alkaloids.⁹ ⁻¹³ Elsewhere we reported that a transformed root culture (AR‐4) grew rapidly and produced scopolamine, but the content was lower than that in untransformed roots (RB‐1).⁹⁰

Repeated selection of highly productive cell lines is an important strategy for the production of useful secondary metabolites by cultured plant cells.¹⁴ ⁻¹⁶ However, differentiated cultures such as root cultures are considered to be more stable than cell suspension cultures, and there are few reports on the efficiency of repeated selection applied to root cultures, and selection is made only at the beginning of root induction.

We report here repeated selection using *A. rhizogenes*‐transformed and untransformed root cultures, and enhancement of the scopolamine content of *Duboisia myoporoides* by the transformed root cultures.

**Materials and Methods**

*Conditions for root culture.* Untransformed root cultures of *Duboisia myoporoides* R. Br. were hormonally induced, and were subcultured at 3‐week intervals in Nitsch and Nitsch (NN) liquid medium⁷ containing 3% sucrose and 10 μg indolebutyric acid (IBA). The inoculum used was about 0.1 g fresh weight (FW) per 100 ml Erlenmeyer flask that contained 20 ml of fresh NN medium. Root cultures were agitated at 25°C and 100 rpm on a rotary shaker with an agitation diameter of 25 mm. In the subculture of the transformed root clones (AR‐4)⁹⁰ the conditions were the same as for the untransformed root cultures except for the absence of IBA.

*Method for selection of untransformed roots.* Untransformed root tips (about 1 cm long) were excised and inoculated into wells (16 mm φ) containing 1 ml of NN liquid medium with 10 μg IBA. After 3 weeks of culture, the roots were transferred to 100 ml Erlenmeyer flasks containing 20 ml of fresh NN medium with 10 μg IBA and cultured for another 3 weeks. A part of the root culture (about 0.1 g FW) was subcultured, and the rest was harvested and dried, after which the alkaloid content was determined. The same procedure was used for subsequent selections.

*Method for selection of transformed roots.* Transformed root tips (about 1 cm long) were excised and inoculated to 20 ml of NN liquid medium that contained no growth regulators. After 2 months of culture, a part of the root culture (about 0.1 g FW) was subcultured. The rest was harvested and dried, after which the alkaloid content was determined. The same procedure was used for subsequent selections.

*Detection of opines.* Root tissue extracts were analyzed for the presence of agropine and mannanope by high voltage paper electrophoresis as described by Petit et al.¹⁸ In brief, 0.1 g of cultured hairy roots was homogenized in an Eppendorf tube with 0.5 ml of distilled water, then centrifuged at 13,000 rpm for 5 min. The extract, as well as standard samples that were synthesized from l‐glutamine and D‐mannose as described by Petit et al.,¹⁸ was spotted on Whatman 3MM paper and electrophoresed at 10 V/cm for 180 min in buffer consisting of formic acid, acetic acid, and distilled water (30:60:910, v/v/v). The dried chromatogram was stained with alkaline silver nitrate reagent.¹⁹

*Extraction and analysis of alkaloids.* The harvested roots (about 50 mg dry weight (DW)) were freeze‐dried, then powdered by crushing them in a test tube, after which they were ultrasonicated for 30 min in 5 ml of 0.05 N H₂SO₄ solution. Homatropine was added as the internal standard, and the solution was made alkaline with 28% NH₄OH. A1‐ml sample was put on an Extrelut‐1 column (Art. 15731, Merck, NJ, U.S.A.) and after 5–10 min, 6 ml of CHCl₃ was put on the column. The CHCl₃ extracts were evaporated to dryness at 30°C.

Abbreviations: IAA, 3‐indoleacetic acid; IBA, indolebutyric acid; NN, Nitsch and Nitsch; FW, fresh weight; DW, dry weight.
For the extraction of alkaloids from the culture medium, 1.0 ml of medium was made alkaline with 100 μl of 28% NH₄OH. Extraction was done with an Extrelut-1 column as described for cell extraction.

The alkaloids obtained were analyzed by GLC (Shimadzu Model GC-9A), using a capillary column CBP-1 (25 m × 0.25 mm id). The column temperature was 260°C, and the flow rate of the N₂ carrier gas was 1.05 ml/min. The split ratio was 1:1, and the detector was an FID. Alkaloid contents were calculated on a dry weight basis.

Results and Discussion

No enhancement of scopolamine content in untransformed roots

The scopolamine content in initiated, untransformed root cultures was 0.95%. Root tips were separated by the described selection procedure and selection was repeated 4 times. Scopolamine contents varied widely among the lines, but the mean values were almost the same (about 1%) for each passage (Fig. 1). As repeated selection was not appropriate for improving scopolamine production in untransformed root cultures, we investigated the repeated selection of transformed root cultures.

Enhancement of alkaloid contents and decrease of growth in transformed roots

The content of scopolamine in initiated AR-4 transformed root cultures was 0.15%. Root tips that were separated by the described selection procedure were cultured for 2 months. The scopolamine contents of the selected lines varied as much as 0.28%. The root culture that had the highest scopolamine content was divided, after which selection was repeated 8 times. The scopolamine content distributions in the transformed root cultures are shown in Fig. 2. The scopolamine contents of the lines obtained at each selection increased with the number of selections, but the distributions gradually became skewed in comparison to those of the parent lines. In the 8th passage, the scopolamine content of the highest-producing root line was 3.2% DW.

At the beginning of selection, lines differed in appearance; e.g., in thickness, length, and lateral branching. After 8 selections, however, only fine root lines with extensive lateral branching remained. The localization of a bifunctional enzyme, hyoscyamine 6β-hydroxylase, that catalyzes hyoscyamine to 6β-hydroxyhyoscyamine and/or scopolamine is in the pericycle of young roots, and the correlation coefficient for its activities and the combined alkaloid content of 6β-hydroxyhyoscyamine plus scopolamine is...

![Fig. 1](image1.png)

**Fig. 1.** Scopolamine Content Distributions in Untransformed Roots from Selections 1 to 4.

The root line that had the highest scopolamine content was used as the parent root in the next selection. X is the average content. Cultured roots complete with tips (about 1 cm long) were inoculated into individual wells containing 1 ml of NN liquid medium and 10 μl IBA and cultured for 3 weeks. The root cultures then were transferred to a 100-ml Erlenmeyer flask containing 20 ml of fresh NN liquid medium and 10 μl IBA and cultured for another 3 weeks. A part of this culture (about 0.1 g FW) was subcultured. The rest was harvested and dried, and the alkaloid content determined.

![Fig. 2](image2.png)

**Fig. 2.** Scopolamine Content Distributions in Transformed Roots from Selections 1 to 8.

The root line that had the highest scopolamine content was used as the parent root in the next selection. X is the average content. Transformed roots complete with tips (about 1 cm long) were excised and inoculated into Erlenmeyers flasks, each containing 20 ml of NN liquid medium without growth regulators. After 2 months of culture, a part of this culture (about 0.1 g FW) was subcultured. The rest was harvested and dried, after which the alkaloid content was determined.

![Fig. 3](image3.png)

**Fig. 3.** Changes in Cell Yield, Alkaloid Contents, and the Alkaloid Ratio in Selected Transformed Root Cultures from Selections 1 to 8 That Had the Highest Scopolamine Contents.

Culture conditions were the same as in Fig. 2. Symbols: cell yield (□); total alkaloid content (○); scopolamine content (●); 6β-hydroxyhyoscyamine ratio (□).
Differentiated cultures such as root cultures show inverse or proportional relations for primary metabolism (such as growth) and the production of secondary metabolites. Nicotine production in tobacco root cultures is proportional to root dry weight; but, hyoscyamine production in *Hyoscyamus albus* root cultures is inverse to it. Our results show that there is no statistical correlation \( r = 0.208 \) for untransformed and \( r = -0.201 \) for transformed root cultures between scopolamine content and growth, as has been reported for *H. niger* root cultures.

**Stability of transformed root cultures**

*A. rhizogenes* strain HRI carries the Ri plasmid in which the TR region has a locus homologous to that of the auxin genes (*tms1* and *tms2*) of Ti T-DNA that encode 3-indoleacetic acid (IAA) synthetase. The slow growth rate is thought to be caused by the deletion of T-DNA during subculture. Agropine and mannoine are the detectable opines in roots transformed with Ri plasmid. To determine whether T-DNA remained, the presence of agropine and mannoine in extracts of transformed roots after 8 selections was examined by paper electrophoresis followed by alkaline silver nitrate staining. Both agropine and mannoine were detected in the extracts of transformed roots but not in those of untransformed roots. As T-DNA was detected in the selected roots, the slow growth rate was not due to its deletion during subculture. We reported elsewhere that the difference between the endogenous IAA contents in transformed and untransformed roots is not sufficient to explain the difference in growth.

White *et al.* (1985) reported that *rol* loci in the TL region are essential for the virulence of *A. rhizogenes* and root growth. The slow growth of the selected transformed roots may be due to the gene products encoded by these loci.

Repeated selection resulted in an increase in the scopolamine content of transformed roots but not in the content of untransformed roots. Why was there a large increase in the scopolamine content of transformed roots? There is considerable variation in transformed roots as a result of the copy number, size, and chromosomal location of T-DNA fragments integrated in the plant genome. One possibility is that the initial root line (AR-4) consisted of heterogeneous cells even though it had been established from a root tip. If so, the highly productive root line obtained by repeated selection must be the result of the removal of the heterogeneous cells.

Mano *et al.* reported that hairy roots of *D. leichhardtii* transformed with *A. rhizogenes* had considerable variation in growth rate, alkaloid content, and productivity from line to line. They established a high scopolamine-producing clone and the productivity of that line was stable for more than 12 successive subcultures. Kamada *et al.* also reported that hairy root cultures of *Atropa belladonna* transformed with *A. rhizogenes* constantly produced atropine and scopolamine under non-selective conditions during a prolonged period. In this study, we first showed that repeated selection was applicable to transformed root cultures for obtaining high scopolamine-producing lines under selective conditions.

The growth rate of transformed root cultures decreased after repeated selection, but it might be improved by addition of auxin to the culture medium or by changing...
the basal culture medium\(^{10-12}\) although alkaloid contents were decreased by them. Therefore, a two-stage culture of scopolamine-rich roots should be useful for the efficient production of scopolamine. In a two-stage culture, roots are propagated for optimal growth then transferred to medium optimal for scopolamine production. Two-stage culture experiments are now in progress to identify the optimum conditions for that type of culture.

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**References**