

## Producing a High Scopolamine Hairy Root Clone in *Hyoscyamus muticus* through Transformation by *Agrobacterium rhizogenes*

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### ABSTRACT

*Agrobacterium rhizogenes* causes hairy root disease in plants. These hairy roots are genetically stable and grow rapidly. Transformed hairy roots of *Hyoscyamus muticus* induced by the bacterium can produce tropane alkaloids in trace amounts of intact plant tissues. In this research, in order to compare growth and biosynthetic stability of hairy roots with wild type ones, leaf and nodal segments of the plant were inoculated with the *A. rhizogenes* strains A4 and LBA9402. When hairy roots appeared, both the wild type and transformed roots were cultured in a liquid B5 medium. The amounts of tropane alkaloids in both the wild type and transformed roots were measured using HPLC. The growth rates of transformed roots were stable and their dry weights were up to four times higher than those of wild type roots. Alkaloid content was the same for both transformed roots and wild type ones. However, when hairy roots underwent an autonomous dedifferentiation and produced callus, their hyoscyamine content decreased considerably, whereas not only did their scopolamine content, not decrease, but in some cases it increased dramatically. A callus producing hairy root clone, produced 2.72 mg/g scopolamine which is a significantly high record for hairy roots of *H. muticus*.

**Keywords:** *Agrobacterium rhizogenes*, Hairy root culture, *Hyoscyamus muticus*, Tropane alkaloid.

### INTRODUCTION

Tropane alkaloids, especially hyoscyamine and scopolamine, are widely used in medicine for their mydriatic, antispasmodic, anticholinergic, analgesic and sedative properties (24). The synthetic production of these alkaloids is more expensive than their extraction from plant materials and they are, therefore, currently industrially extracted from various solanaceous plants belonging to the genera *Atropa*, *Duboisia*, *Datura* and *Hyoscyamus*. During the past few years, considerable efforts have been made to develop an economically feasible method of *in vitro* production of these compounds.

Different species of *Hyoscyamus* are rich sources of tropane alkaloids. Amongst different species of *Hyoscyamus* (11 species) the alkaloid content in *H. muticus* is the highest and the plant is an important source of these alkaloids. Much research has, therefore, been conducted on this plant to discover a suitable alternative method for producing tropane alkaloids through *in vitro* procedures, including callus and suspension cultures, protoplast cultures, somatic hybridization and root cultures (6, 12, 13, 14, 15 and 16). Tropane alkaloids are synthesized in roots and then transported to the aerial parts of the plant (14). In 1986, promising results were obtained from wild type root cultures of the species *Hyoscyamus* (3).

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Tropane alkaloids that are scarcely synthesized in undifferentiated cells were produced at relatively high levels in cultured roots. However, the main problem with wild type roots is usually their slow growth rate (3).

Almost at the same time, transformed hairy roots were generated in some tropane alkaloid producing plants through transformation by *Agrobacterium rhizogenes*, and the possibility of alkaloid production in cultures of these transformed roots was studied (5). *A. rhizogenes* is able to transfer a part of its DNA (T-DNA), carried on a large plasmid (Ri plasmid), to the genome of the host plant (8). Integration and expression of T-DNA genes in the host plant cells leads to the development of hairy roots which can be excised and grown *in vitro* as hairy root cultures. These transformed roots have received considerable attention from plant biotechnologists for the production of secondary plant compounds. Hairy roots are highly branching and can be grown on a hormone-free medium (21). These fast growing hairy roots are genetically stable and possess the whole biosynthetic potential of wild type roots. Moreover, transformed roots are able to regenerate whole viable plants and maintain their genetic stability during further subculturing and plant regeneration (2, 11, 19, and 20).

For the first time, the hairy root cultures of *H. muticus* were established by Flores *et al.* (1987). They reported that hairy roots produce tropane alkaloids at levels which are often comparable to, or greater than, those of the intact plants (17). So, hairy root cultures of *H. muticus* were discussed as promising systems for the *in vitro* production of tropane alkaloids. Since that time, the production of tropane alkaloids and polyamines by hairy roots of *H. muticus* has been extensively studied by Sevon *et al.* (2001), Oksman-Caldentey *et al.* (1996) and some other research groups (3, 4, 6 and 9).

The susceptibility of plant species to *Agrobacterium* strains varies greatly. Vanhala *et al.* (1995) showed that the most virulent *Agrobacterium* strains on *H. muticus* are *A. tumefaciens* strain C58 and *A. rhizogenes*

strain LBA9402. They reported that the used *Agrobacterium* strain, has a significant influence on the phenotype as well as on the growth rate and hyoscyamine content of the root culture clones. Sevon *et al.* (1998), chose four different hairy root clones of *H. muticus* for long term study. They reported that tropane alkaloid production in hairy roots was the highest in the late stationary phase and that hyoscyamine was the main alkaloid. The fastest growing clones produced the lowest alkaloid level and the slowest growing clones produced the highest. Furthermore, extremely fast growing clones, in some cases, underwent dedifferentiation and produced calli. This event was strongly correlated with a decrease in secondary product formation (17).

As stated above, the characteristics of hairy roots induced by *A. rhizogenes* vary, from clone to clone. They are influenced by the type of plant, *Agrobacterium* strain, type of explant and the experimental conditions. This experiment, therefore, was conducted to create new combinations of desirable characteristics in term of growth rate and hyoscyamine and scopolamine content.

## MATERIALS AND METHODS

### Bacterial Strains

*A. rhizogenes* agropine strains A4 and LBA9402 (kind gifts from Dr. Oksman-Caldentey, VTT Biotechnology, Finland) were used in this study. For the transformation experiments, 48 hours old bacterial suspension, OD= 0.6 – 0.8 in 590 nm, grown in liquid YMB medium at  $28 \pm 2$  °C on a rotary shaker at 100 rpm, was used (24).

### Plant Materials

Seeds of *H. muticus* L. strain Cairo (courtesy of Dr. Oksman-Caldentey) were surface sterilized using 5% sodium hypochloride for five minutes, rinsed with sterile distilled water four times and germinated on a basal MS

medium. After two weeks, the small seedlings were transferred onto a pasteurized light soil in two kg pots and grown in a greenhouse for a further four weeks at a temperature of  $26 \pm 2$  °C at a photoperiod of 16 h light/8 h darkness. Leaf and nodal segments of six week-old plants were used for bacterial inoculation.

### Transformation and Establishment of Hairy Root Cultures

Leaf and nodal segments (about 1.5-2 cm for leaf and 0.5-1 cm length for nodal segments) were isolated from six-week old plants which were grown in a greenhouse. The explants were surface sterilized using 5% sodium hypochloride for four minutes, rinsed with sterile water five times and used for bacterial inoculation. Leaf segments with the lower epidermis upwards and nodal segments were placed on an agar-solidified, hormone-free LS medium and inoculated with *A. rhizogenes* strains A4 and LBA9402 by wounding the leaves on the midrib and nodal segments on the side with a sterile hollow needle loaded with a 48 hour-old bacterial suspension. Cultures were incubated at  $26 \pm 2$  °C in a photoperiod cycle of 16 h light/8 h darkness. After two days of inoculation, the explants were transferred to fresh media containing 500 mg/L cefotaxime to eliminate bacteria and further subculturing were performed two times at four-day intervals to complete the elimination of bacteria. When roots appeared, each single root was transferred to 25 ml of B5 liquid medium containing 25 g/L sucrose in 100 ml conical flasks and grown at  $26 \pm 2$  °C on a gyratory shaker (90 rpm) in darkness.

For growth analysis, two of the fastest growing and two of the slowest growing LBA9402 clones were selected and five cultures established from each clone (A4 clones were not successful). For each culture, 10 root tips of 2.5-3.5 cm in length were transferred to the B5 liquid medium and incubated under the same conditions as mentioned above. The same manner was fol-

lowed for the establishment of wild type root cultures, but the root tips were cut from two week-old sterile seedlings. The cultures were subcultured at four-week intervals for seven months. Each subculture was established in the same manner and the biomass produced by each culture was harvested and washed twice with sterile distilled water and lyophilized. Dry root material was used for alkaloid analysis.

### Detection of Bacterial DNA in Hairy Roots

Total DNA samples were isolated from 24 hour-old cultures of *A. rhizogenes* strains A4 and LBA9402 using a DIAAtom™ DNA Prep Kit. DNA was isolated also from hairy roots and from the control roots according to Dellaporta *et al.* (1983). The polymerase chain reaction was used to confirm the presence of the *rolB* and *rolC* genes in the hairy roots. Oligonucleotide primers for the PCR detection of homologous sequences to *rolB* and *rolC* genes were selected according to Krolicka *et al.* (2003) (7). The homology of selected primer sequences to *rolB* and *rolC* genes were confirmed using program BLAST at NCBI. Primer sequences were as below:  
*rolB*: 5'- GCTCTTGCAGTGCTAGATTT-3' and 5'- GAAGGTGCAAGCTACCTCTC-3'  
*rolC*: 5'- CTCCTGACATCAAACCTCGTC-3' and 5'- TGCTTCGAGTTATGGGTACA-3'.

Each PCR reaction contained: standard PCR buffer (Cinnagen Inc., Iran), 1.0 U Taq DNA polymerase (Cinnagen Inc., Iran), 1.0 µl MgCl<sub>2</sub> (50 mM), 0.5 µl dNTP mix. (10 mM), 8 pmol of each primer and 30–60 ng of the target DNA (final volume 25 µl). Amplification conditions were: 40 cycles, initial denaturation at 94 °C for 3 minutes, denaturation at 94 °C for 1 minute, primer annealing at 54 °C for 1 minute, elongation at 72 °C for 1 minute, followed by final elongation for 6 minute. The amplified fragments were separated by electrophoresis in a 1.2% agarose gel in TBE buffer. The gel was stained with ethidium bromide and ob-



served under UV light.

### Alkaloid Extraction and HPLC Analysis

For alkaloid extraction, 50 mg of powdered plant material were soaked in 8 ml  $\text{CHCl}_3$ -MeOH- $\text{NH}_4\text{OH}$  (15:5:1 v/v/v) for 6 hours with vortex mixing at 60 minute intervals. The supernatant was filtered and the residue was extracted twice with the aforementioned solution with sonication for 20 minutes and mixed with a vortex at five minutes intervals. After centrifugation (5000 rpm, 10 minutes), the supernatants and filtrate were collected and evaporated under reduced pressure to dryness. The alkaloid extracts were dissolved in 1 ml mobile phase and 20  $\mu\text{l}$  were injected into HPLC. A TO-SOH ODS-120T column (4.6 i.d.  $\times$  250 nm) was used, kept at 40  $^\circ\text{C}$  and eluted isocratically with MeCN-10 mM SDS (pH 3.3, adjusted with 1%  $\text{H}_3\text{PO}_4$ ) 2:3 (9 and 22). The flow rate was 1.1 ml/min. throughout. The effluent was detected by UV at 215 nm. For each alkaloid, two standard solutions were prepared and analysed, 12.5 and 75 mg/L for scopolamine and 25 and 150 mg/L for hyoscyamine. Regression functions were calculated according to pick area amounts

presented for standard solutions by HPLC set. The amounts of hyoscyamine and scopolamine in the root extracts were calculated through inserting the pick area amount of each sample in to the regression functions.

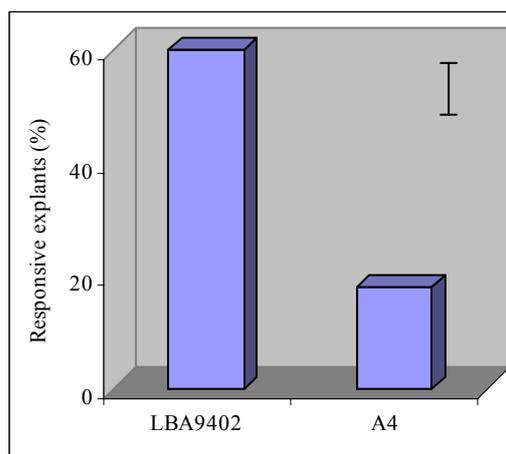
## RESULTS

### Establishment of Hairy Root Cultures

Hairy roots appeared at the wound sites of explants 12-37 days after infection with *Agrobacterium*. These roots were fast growing, highly branching, plagiotropic, and hairy in morphology and showed hormone autotrophy (Figure 1). Figure 2 shows the average of responsive explants to LBA9402 and A4 strains. The transformation frequency was three times higher with LBA9402 compared to the A4 strain. When the root tips were transferred to liquid B5 medium without plant growth regulators, they failed to grow for 5-7 days and then began to grow rapidly and produced many branches. Small calli appeared in old parts of hairy root clones which can be the result of changes in endogenous hormones, caused by T-DNA induction or local wounding due to shaking. Although hairy root clones were



**Figure 1.** Appearance of hairy roots in inoculation sites of the nodal segment (A) and leaf segment (B) of *H. muticus* inoculated with *A. rhizogenes* strain LBA9402.



**Figure 2.** Comparison of explants responsive to *A. rhizogenes* strains: A4 and LBA9402. Means of 4 replicates (The Bar is LSD 0.05).

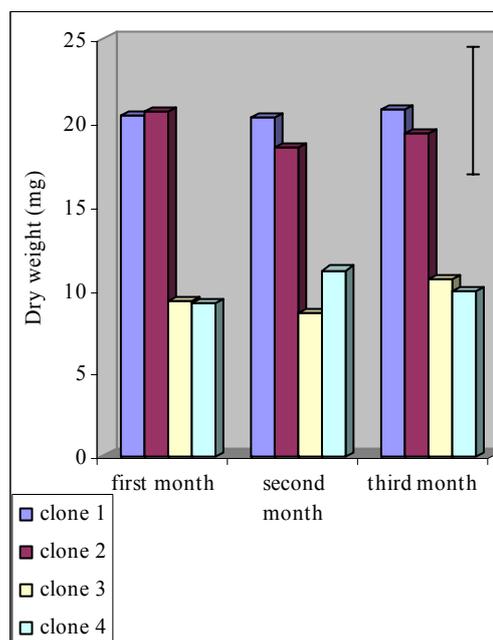
obviously fast growing, there was a considerable difference between the growth rates of different clones and some grew more rapidly than others. However, LBA9402 clones grew faster than A4 clones, so that a considerable biomass was produced by each LBA9402 clone after 28 days (Figure 3), whereas A4 clones grew very slowly. Hence, the two fastest growing and the two slowest growing LBA9402 clones were selected for further studies.



**Figure 3.** The fast growing LBA9402 hairy root clone, C1, in a liquid B5 medium.

### Comparison of Growth Rate and Stability

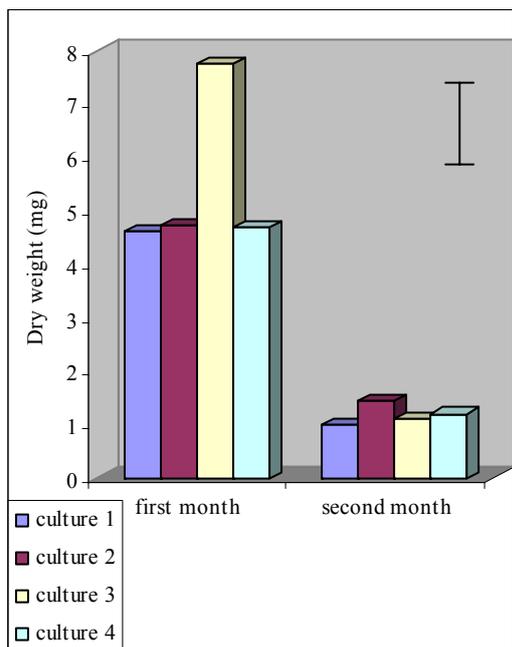
Although, the growth rate of each clone varied some what in different subculture periods, but the differences were not statistically significant. So the growth rate of hairy root clones remained stable for seven months (Figure 4). As discussed earlier, Sevón *et al.* (1998) have also reported considerable growth stability of hairy root clones of *H. muticus* over six years (14).



**Figure 4.** Comparison of the growth rates of selected hairy root clones in different subculture periods. Means of 3 replicates (The Bar is LSD 0.05).

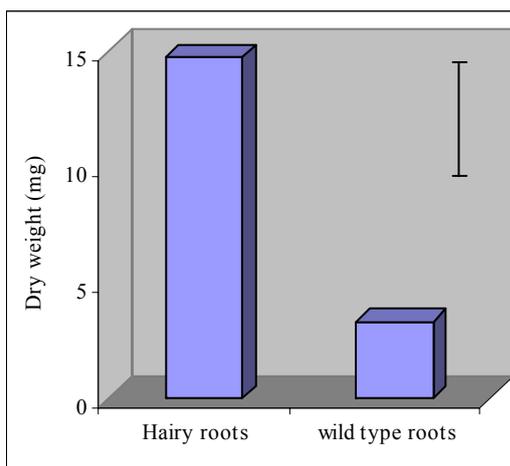
Wild type roots also grew in a hormone-free medium. However, the growth rate of these roots was significantly lower than that of hairy roots. Wild type roots did not grow stably such that their growth rate decreased to half in the second month and stopped by the third month (Figure 5).

Hairy root clones also grew more rapidly and produced more lateral branches than wild type roots. The slowest growing hairy root clone grew two times faster than the fastest growing wild type root culture and



**Figure 5.** Comparison of the growth rates of wild type root cultures in different subculture periods. Means of 3 replicates (The Bar is LSD 0.05).

this rate was more than four times for the fastest growing hairy root clone (Figure 6).



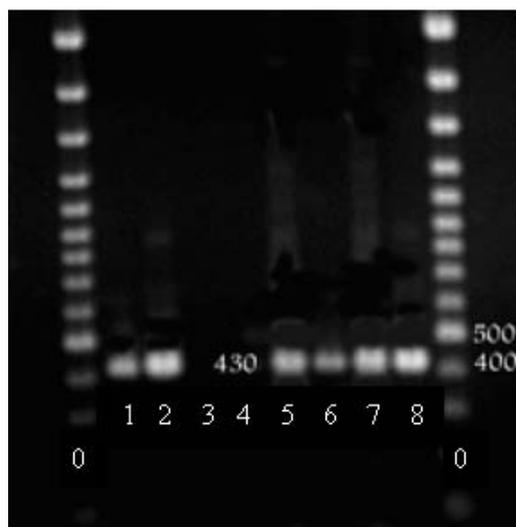
**Figure 6.** Comparison of the growth rates of hairy root clones with wild type root cultures after two months. Means of 4 replicates (The Bar is LSD 0.05).

### Detection of *Rol* Genes in Hairy Roots

The polymerase chain reaction, with primers designed to amplify specific fragments of the *rolB* and *rolC* genes of *A. rhizogenes*, was used to confirm integration of T-DNA into the genome of hairy roots. Amplification with *rolB* primers showed a 430 bp band (Figure 7) and when *rolC* primers were used, a 612 bp band (Figure 8) was visualized for DNA isolated from both hairy roots and *A. rhizogenes* strains. The PCR products were absent in reactions performed with DNA isolated from wild type roots.

### Comparison of Alkaloid Content

Based on standard solutions, the retention time for scopolamine (Figure 9) and hyoscyamine (Figure 10) were 15 and 19 minutes, respectively. Comparison of the



**Figure 7.** PCR analysis of *rolB* genes of *A. rhizogenes*, (0): Size marker (GeneRuler™ 100bp DNA Ladder Plus), (1): Positive control, amplified band from the DNA of LBA9402 strain, (2): Positive control, amplified band from the DNA of A4 strain, (3): Water as control; (4): The band is absent in reaction with wild type root DNA, (5 and 6): Amplified band from the DNA of hairy roots induced by LBA9402 strain, (7 and 8): Amplified band from the DNA of hairy roots induced by A4 strain.



**Figure 8.** PCR analysis of *rolC* genes of *A. rhizogenes*, (0): Size marker (GeneRuler™ 100bp DNA Ladder Plus), (1): Positive control, amplified band from the DNA of LBA9402 strain, (2): Positive control, amplified band from the DNA of A4 strain, (3): Water as control; (4): The band is absent in reaction with wild type root DNA, (5 and 6): Amplified band from the DNA of hairy roots induced by LBA9402 strain, (7 and 8): Amplified band from the DNA of hairy roots induced by A4 strain.

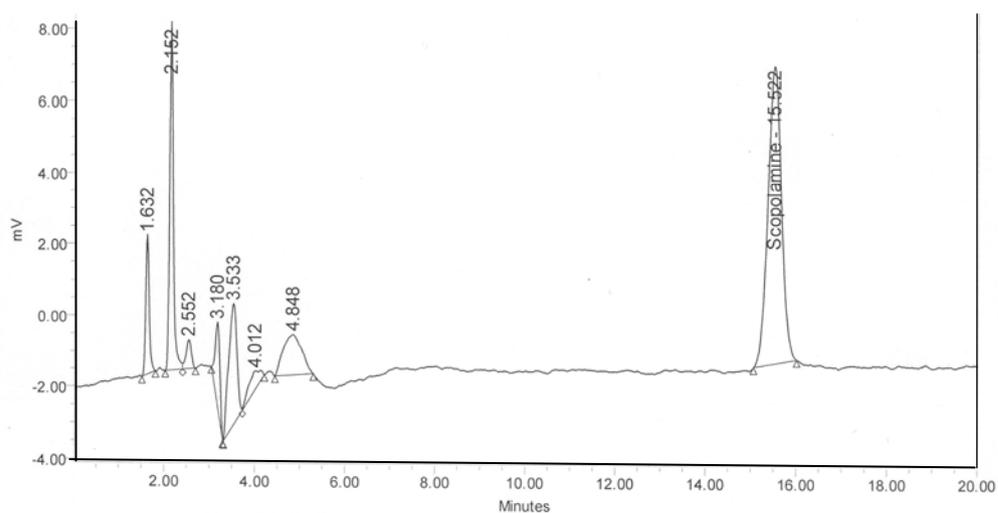
alkaloid content as one milligram alkaloid per gram dry root material did not show any significant difference between hairy roots and wild type ones. Previous studies on hairy roots of *H. muticus* had also shown that there was no considerable difference between the alkaloid contents in hairy roots and wild type roots (17).

The hyoscyamine and scopolamine contents in callus producing hairy roots were also compared with both the common hairy roots and wild type roots of the plant. This comparison represented a considerable variation for the hyoscyamine, but not for scopolamine, contents in callus producing hairy roots with both common hairy roots and wild type roots. The maximum hyoscyamine content in a hairy root clone was 5.4 mg/g for the fastest growing clone, C1 (Figure 11) and for wild type roots and callus producing hairy roots was 6 mg/g

(Figure 12) and 1.26 mg/g (Figure 13), respectively. The maximum scopolamine content in a hairy root clone was 0.68 mg/g for the fastest growing clone, C1 (Figure 11) and for wild type roots and callus producing hairy roots was 0.7 mg/g (Figure 12) and 2.72 mg/g (Figure 13), respectively. Comparison of the hyoscyamine and scopolamine contents in three root types is shown in Figures 14 and 15 respectively.

## DISCUSSION

It seems that the response of *H. muticus* to different strains of *A. rhizogenes* differs, since LBA9402 was more successful than A4 in its transformation. Different strains had a different influence on the phenotype and growth rate of hairy root clones. Since the phenotype and growth rate of hairy root clones are very important factors in selecting a clone for further study to achieve commercial production of tropane alkaloids *in vitro*, one must try to select the most effective strain of *A. rhizogenes* to induce hairy roots. Significant difference in the growth rate of LBA9402 and A4 induced hairy root clones could be due to the difference in the copy number of inserted T-DNAs into the genome of different clones or the difference in expression patterns of T-DNA genes in different hairy root clones (2). Since the growth rate and tropane alkaloid production of individual hairy root clones was stable, this variation is highly valued for selecting fast growing and highly producing ones. The genetic stability of hairy roots of *H. muticus* has two advantages. Firstly, if we can achieve commercial production of tropane alkaloids *in vitro*, then we can use our producing biomass for a long period without a significant decrease in yield. Secondly, it is possible to obtain stable hairy roots containing some new genes to achieve new purposes. New genes could be transferred to hairy roots with the purpose of increasing their growth rate, producing new metabolites or a change in the pattern of alkaloid production (4).

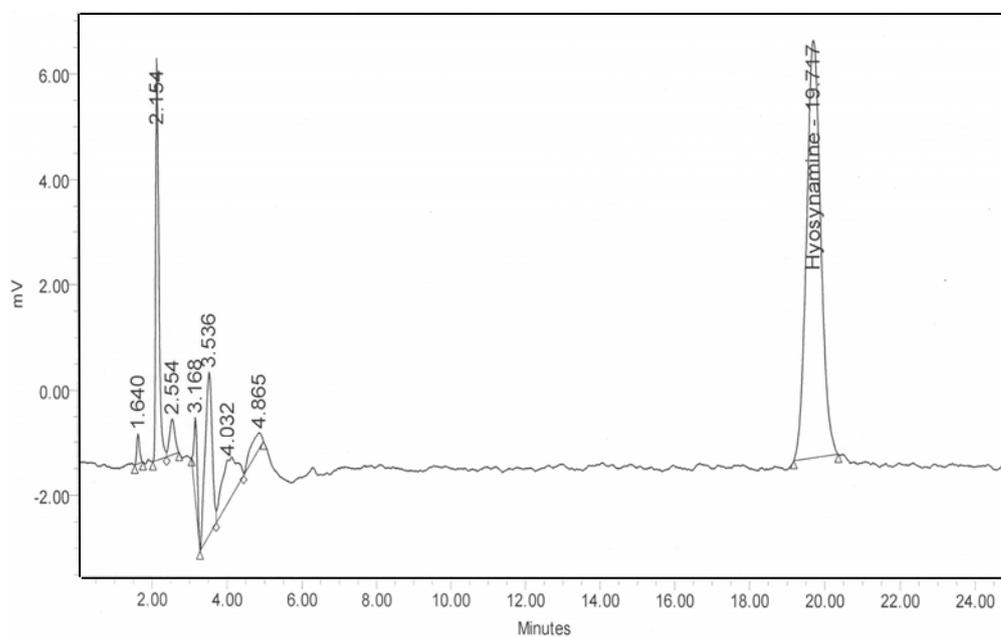


Peak no.	Peak name	Rt (min.)	Area ( $\mu\text{V}\cdot\text{sec}$ )	%Area	Height ( $\mu\text{V}$ )	%Height
8	Scopolamine	15.552	172122	48.22	8341	27.73

**Figure 9.** HPLC diagram for standard scopolamine ( $12.5 \text{ mgL}^{-1}$ ).

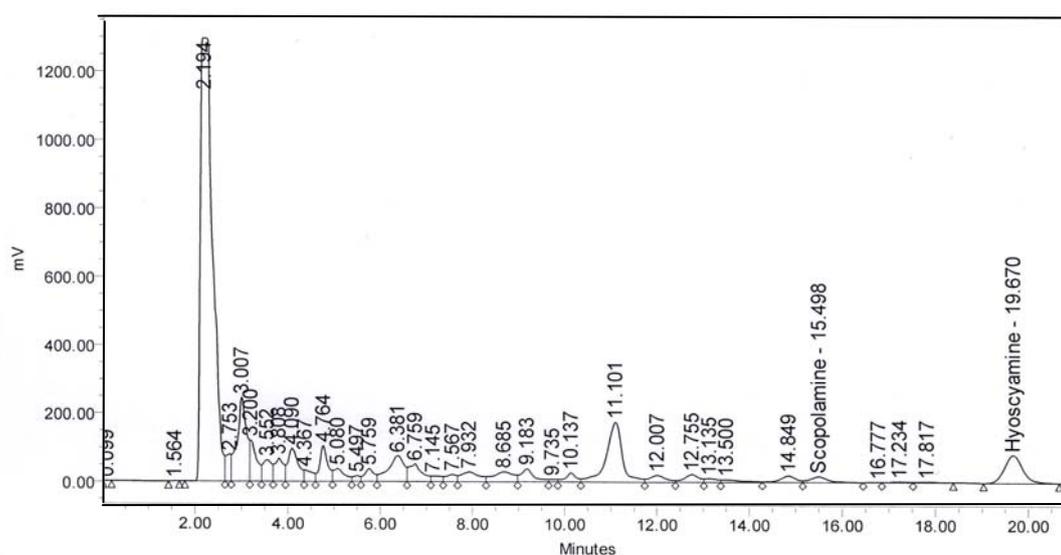
Two major factors which influence *in vitro* production of secondary metabolites are the amount of biomass produced over a certain

time span (growth rate) and the amount of metabolites synthesized per unit of biomass (biosynthesis rate). As in previous studies,



Peak no.	Peak name	Rt (min.)	Area ( $\mu\text{V}\cdot\text{sec}$ )	%Area	Height ( $\mu\text{V}$ )	%Height
8	Hyoscyamine	19.717	208301	60.74	7931	35.08

**Figure 10.** HPLC diagram for standard hyoscyamine ( $25 \text{ mgL}^{-1}$ ).



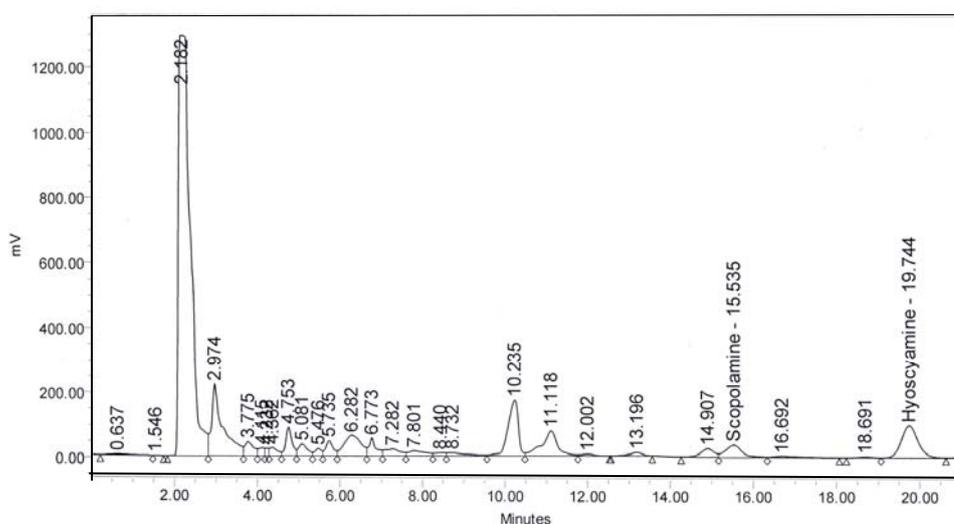
Peak no.	Peak name	Rt (min.)	Area ( $\mu\text{V}\cdot\text{sec}$ )	%Area	Height ( $\mu\text{V}$ )	%Height
30	Scopolamine	15.498	470658	0.92	16816	0.59
34	Hyoscyamine	19.670	2249746	4.41	81586	2.86

**Figure 11.** HPLC analysis of the hairy root clone containing maximum content of scopolamine and hyoscyamine.

the results of this study showed that the growth rate of *H. muticus* hairy roots is higher than that of wild type roots. Comparison of the alkaloid contents of hairy roots with wild type ones did not reveal any significant difference and there was no correlation between growth and biosynthetic rates. It is obvious that the best situation is when an increase in growth rate is not correlated with decreasing of alkaloid content. As this fact was observed in case of *H. muticus* hairy roots, it represents them as a valuable system for producing tropane alkaloids *in vitro*.

Sevon *et al.* (1998) reported that an increase in the growth rate of a hairy root clone is correlated with a considerable decrease in its tropane alkaloid content (17). In contrast, we observed that the fast growing hairy roots grew four times more than wild type root cultures without any decrease in their biosynthetic ability. This means that from a certain amount of initial biomass of

hairy roots we can obtain four times more tropane alkaloids than the same amount of wild type roots. On the other hand, when hairy roots formed callus, their hyoscyamine content decreased strongly, whereas their scopolamine content not only did not decrease but also, in some cases, it increased surprisingly. These results can complete the report of Sevon *et al.* (1998), who reported, "when hairy roots form callus, change in root morphology towards an undifferentiated state is correlated strongly with a decrease in tropane alkaloid production". However, these results may not indicate that there is a certain correlation between callus production and increasing the scopolamine content in hairy roots, as the difference between callus producing hairy roots with common hairy roots was not statistically significant. It is obvious that there are many different factors influencing scopolamine production in callus producing hairy root clones. So far, these factors have not been investigated and



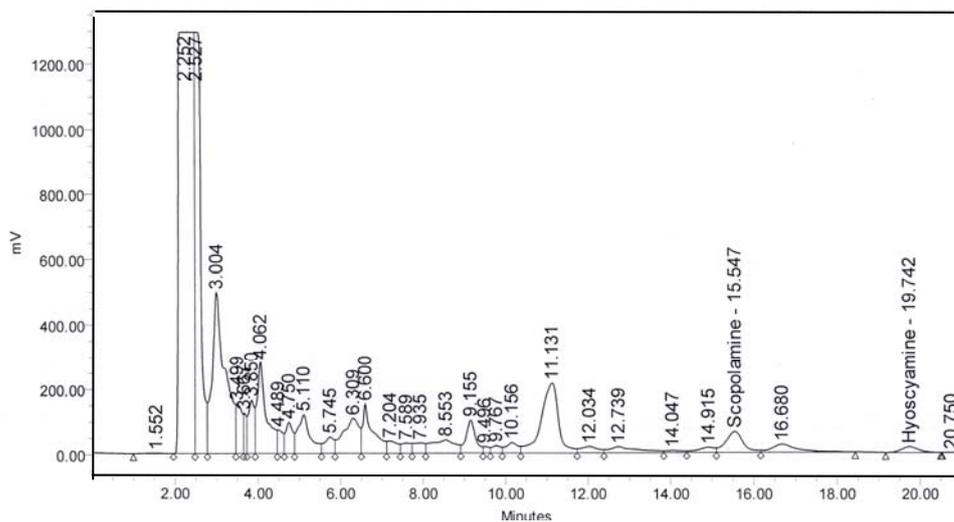
Peak no.	Peak name	Rt (min.)	Area ( $\mu\text{V} \cdot \text{sec}$ )	%Area	Height ( $\mu\text{V}$ )	%Height
24	Scopolamine	15.535	1047664	2.19	37480	1.54
27	Hyoscyamine	19.744	2751543	5.74	99154	4.06

**Figure 12.** HPLC analysis of the wild type root culture containing maximum content of scopolamine and hyoscyamine.

they need further study to be revealed.

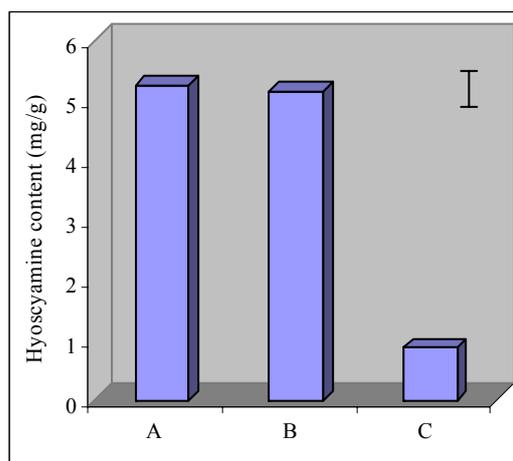
An important challenge concerning *H. muticus* hairy roots is the scopolamine content

of the roots which is considerably lower than the hyoscyamine content, while, scopolamine is the more valuable alkaloid and

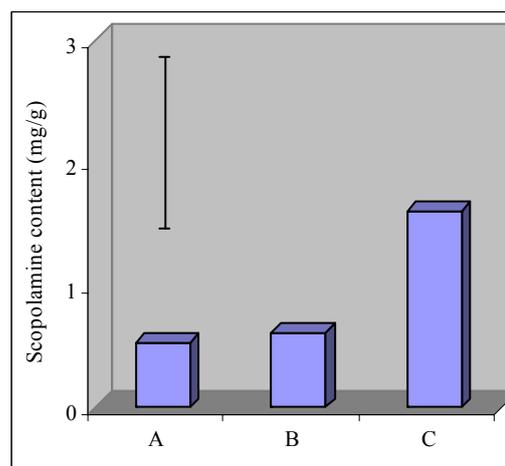


Peak no.	Peak name	Rt (min.)	Area ( $\mu\text{V} \cdot \text{sec}$ )	%Area	Height ( $\mu\text{V}$ )	%Height
28	Scopolamine	15.547	1874083	2.03	64457	1.27
30	Hyoscyamine	19.742	525136	0.57	18033	0.35

**Figure 13.** HPLC analysis of the callus producing hairy root clone containing maximum content of scopolamine and hyoscyamine.



**Figure 14.** Comparison of the hyoscyamine contents in common hairy root clones (A), Wild type root cultures (B) and Callus producing hairy root clones (C). Means of 2 replicates (The Bar is LSD 0.05).



**Figure 15.** Comparison of the scopolamine contents in common hairy root clones (A), Wild type root cultures (B) and Callus producing hairy root clones (C). Means of 2 replicates (The Bar is LSD 0.05).

world demand for this alkaloid is about ten times more than that for hyoscyamine (17). Since, in the biosynthetic pathway of tropane alkaloids, hyoscyamine is converted to scopolamine by hyoscyamine-6- $\beta$ -hydroxylase, there is greater attention paid to increasing the level of scopolamine in hairy roots of tropane alkaloid producing plants as well as intact plants through genetic engineering as performed by Zhang *et al.* (2004). Since the maximum scopolamine content which has been reported in common hairy roots of *H. muticus* is 1 mg/g (17), thus obtaining a callus producing hairy root clone which produces 2.72 mg/g scopolamine (Figure 13) is promising.

#### ACKNOWLEDGEMENTS

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تولید یک کلون ریشه موئین با محتوی اسکوپولامین زیاد در گیاه *Hyoscyamus muticus*  
از طریق تراریختی توسط *Agrobacterium rhizogenes*

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### چکیده

باکتری خاکزی گرم منفی *Agrobacterium rhizogenes* عامل بروز بیماری ریشه موئین در گیاهان میزبان است. این ریشه‌های موئین از نظر ژنتیکی پایدار بوده و دارای رشد سریعی می‌باشند. ریشه‌های موئین تراریخت گیاه بذرالبنج مصری (*Hyoscyamus muticus*) از قابلیت تولید آلکالوئیدهای تروپانی به مقادیر تقریباً برابر با ریشه‌های گیاهان سالم برخوردارند. در این تحقیق برای مقایسه قابلیت‌های رشدی و بیوسنتزی ریشه‌های موئین این گیاه با ریشه‌های نرمال آن، قطعات برگ‌گی و گره‌ای گیاه با باکتری *A. rhizogenes* نژادهای A4 و LBA9402 تلقیح گردید. پس از آنکه ریشه‌های موئین ظاهر شدند، هر دو نوع ریشه‌های نرمال و تراریخت در محیط کشت مایع B5 فاقد هورمون رشد کشت گردیدند. مقدار آلکالوئیدهای تروپانی در نمونه‌های برداشت شده، با استفاده از HPLC اندازه‌گیری شد. سرعت رشد ریشه‌های موئین در دوره‌های واکشت انجام شده، ثابت ماند و بیوماس تولید شده توسط این ریشه‌ها در مقایسه با ریشه‌های نرمال تا ۴ برابر افزایش نشان داد. معذالک وقتی ریشه‌های موئین تحت فرایند تمایززدایی خودبخودی قرار گرفته و کالوس تولید کردند، محتوی هیوسامین آنها بنحو قابل ملاحظه‌ای کاهش یافت، در حالیکه مقدار اسکوپولامین آنها نه تنها کاهش نشان نداد بلکه در بعضی موارد با افزایش فوق العاده‌ای همراه بود. یک کلون ریشه موئین تولیدکننده کالوس، ۲/۷۲ mg/g اسکوپولامین تولید کرد که در مقایسه با ۰/۷ mg/g برای بهترین کشت ریشه‌های نرمال، یک رکورد بی‌نظیر و امیدبخش برای ریشه‌های موئین *H. muticus* می‌باشد.