ACTA BIOLOGICA CRACOVIENSIA Series Botanica 47/1: 101–107, 2005

A STRATEGY FOR OVERACCUMULATION OF SCOPOLAMINE IN *DATURA INNOXIA* HAIRY ROOT CULTURES

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Received November 20, 2004; revision accepted April 18, 2005

Hairy root culture plays an important role in investigation of alkaloid production in culture in vitro. The complexity of scientific work and the production capacity limitations associated with whole plant systems and unorganized cells can be overcome using culture of hairy roots. This paper presents an example of a strategy to produce secondary metabolites from plants, in which we investigated the alkaloid pathway, modified gene expression, and cultivated and optimized hairy root growth in a bioreactor. *Datura innoxia* whole plant was transformed by *Agrobacterium rhizogenes*, and the obtained hairy roots were studied for their tropane alkaloid production. Optimization of medium led to an increase of scopolamine production in hairy root culture of *Datura innoxia* by increasing the produced biomass, but genetic engineering seems to be the best way to increase the accumulation of scopolamine. These root cultures were not able to stably overaccumulate scopolamine. The involvement of putative enzymatic regulation of the tropane alkaloid pathway is discussed.

Key words: Hairy roots, *Datura innoxia,* tropane alkaloid, bioreactor, genetic engineering, *h6h* gene.

INTRODUCTION

Plants are a source of abundant biochemical and therapeutic molecules. Some of them, such as secondary metabolites, can be produced by biotechnology. The production of these molecules has become an active field of research (Sato et al., 2001); it is considered a promising direction for industrial-scale production. In vitro culture of plant cells or tissues presents a number of advantages over traditional culture (Ramachandra Rao and Ravishankar, 2002). It allows in situ extraction and purification of the produced molecules. Hairy roots obtained from transformation of plants by *A. rhizogenes* provide appreciable amounts of biological material for the production of secondary metabolites in bioreactors (Hamill et al., 1987; Wilson, 1997). Hairy roots are stable and can grow rapidly in an adapted culture medium, with no need for plant growth regulators (Shanks and Morgan, 1999; Boitel-Conti et al., 2000; Giri and Narasu, 2000). Genetically transformed hairy roots not only produce similar high-value molecules of interest, but can perform at higher efficiencies than non-transformed roots (Kamada et al., 1986; Flores et al., 1999; Giri and Narasu, 2000).

In Solanaceae, the production of tropane alkaloids by hairy root culture has been reported for several genera including *Atropa*, *Datura*, *Duboisia*, *Hyoscyamus* and *Scopolia*. In most cases, hyoscyamine is the major alkaloid (0.5–0.8 g/100 g dry weight). However, for industrial use, scopolamine is the more valuable of these two alkaloids. Thus there is increasing interest in obtaining cultures with enhanced content of scopolamine.

One way to improve alkaloid accumulation is to improve the producing biomass. Since each line is different, the basic nutrient requirements for each line may vary, and thus growth and production conditions should be optimized individually. Much work has been done in designing bioreactors and developing processes for plant cell cultures (Doran, 1997). In our laboratory we use different bioreactor designs in order to find a set of reliable conditions for optimized production of tropane alkaloids.

Another way to improve alkaloid accumulation in hairy root cultures is to use genetic engineering. Among the few genes available in the tropane alkaloid pathway is the gene encoding the hyoscyamine 6β-hydroxylase (H6H) enzyme, responsible for transformation of hyoscyamine to scopolamine by a two-step reaction (Fig. 1); introducing this gene in transformed roots of different plants leads to greatly enhanced scopolamine production (Hashimoto et al., 1993; Jouhikainen et al., 1999).

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PL ISSN 0001-5296

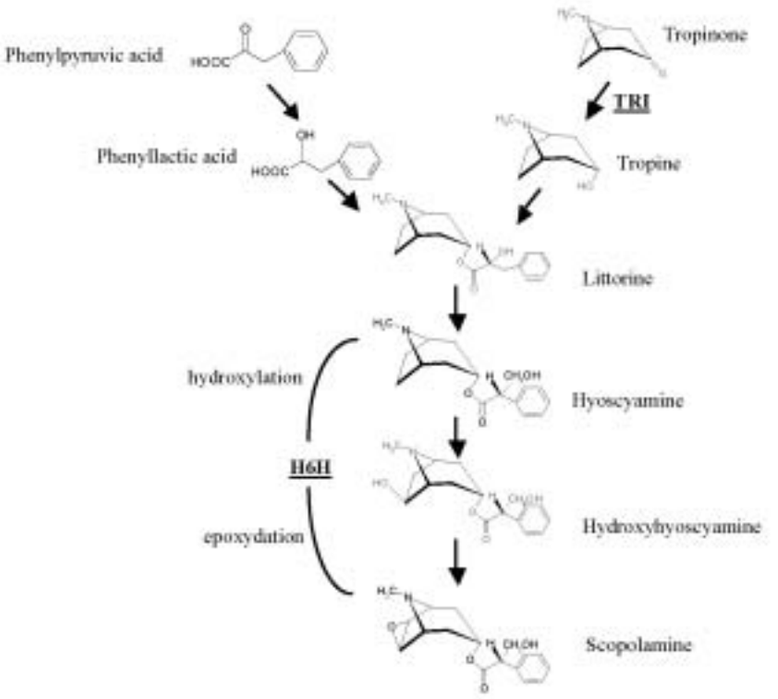

Fig. 1. The tropane alkaloid pathway: last reactions leading to the formation of hyoscyamine and scopolamine. H6H – Hyoscyamine 6β-Hydroxylase; TRI – Tropinone Reductase I.

These two strategies have been studied in our laboratory in *Datura innoxia* hairy root cultures. Here we discuss the implications of the results of both lines of research for improvement of tropane alkaloid production in this plant.

MATERIALS AND METHODS

HAIRY ROOT CULTURES

Plants of *Datura innoxia* Mill. were used in this work to produce hairy root cultures. The transformation was accomplished with *Agrobacterium rhizogenes* strain 1855 containing the pLAL21 construction (Jouhikainen et al., 1999). In the T-DNA of this plasmid, the gene coding for H6H from *Hyoscyamus niger* is cloned under control of the CAMV 35S promotor. The lines obtained by this transformation are cultivated in 250 ml flasks containing liquid B5 medium supplemented with 3% sucrose as described in Lanoue et al. (2004). The control line (C) results from transformation with the same construction but without the *h6h* gene.

CULTURES IN BIOREACTORS

The heart of the experimental setup is a 5 l bubbling reactor with temperature and pH controls, dissolved oxygen measurement and other necessary peripherals (Fig. 2). A second tank is employed for daily measurement of root volume, as discussed below. A computer program runs the supervision and control aspects of each batch. The results are saved and processed in a Microsoft Excel© environment. The liquid phase containing sugar, minerals and other additives is analyzed by sampling and ex situ measurements.

Hairy roots are first cultured in 250 ml Erlenmeyer flasks as described by Boitel-Conti et al. (1995, 1996) in 100 ml Gamborg's B5 culture medium supplemented with 30 g/l sucrose. The reactor is then autoclaved with 4 l culture medium and cooled to 27°C. Inoculation is done under aseptic conditions with 10 g (fresh weight)/l of root

TABLE 1. Root growth properties

At inoculation (day 0)	Initial fresh weight (X_i)	41.8 g
After 20 days	Final fresh weight (X_f)	643.7 g
	Growth ratio = X_f / X_i	15.4
	Production of tropane alkaloids	3.21 g
	Hyoscyamine	2.89 g
	Scopolamine	0.32 g

X_i – Initial biomass; X_f – Final biomass.

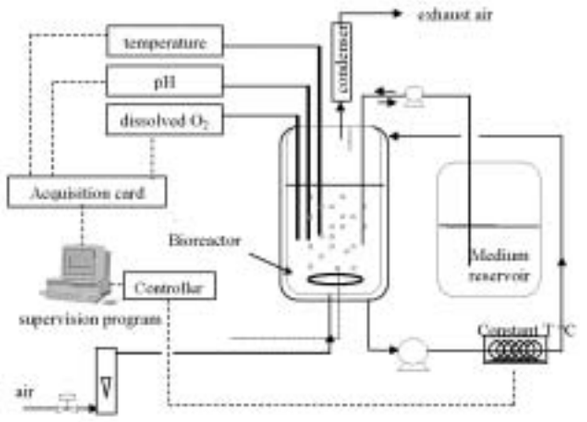

Fig. 2. Bioreactor and its peripherals.

Fig. 3. Bioreactor (5 l) filled with hairy roots after 20 days.

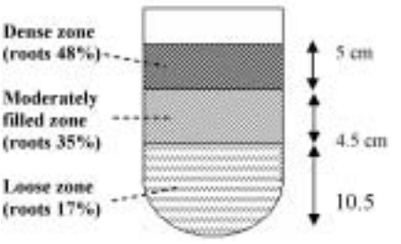

Fig. 4. Zones of root occupation in the bioreactor volume.

tips 20 mm long. The pH is adjusted to 5.8 and the temperature is maintained at 27˚C +/–1.

TROPANE ALKALOID QUANTIFICATION

Biochemical analyses were performed in this work. Scopolamine, hyoscyamine and littorine were quantified by LC/UV (Boitel-Conti et al., 2000; Lanoue et al., 2004) and calculated as mg/100 g DW of each alkaloid. In this way, accumulation in the different lines could be compared.

MOLECULAR ANALYSIS

The hairy root phenotype was confirmed by the presence of *rol* genes in the root genome, using PCR technique. The presence of the *rolA* gene present in the pRi1855 T-DNA was investigated in all lines using the primer CAGAATGGAATTAGCCGGACTA and reverse CGTATTAATCCCGTAGGTTTGTTT. The PCR cycle was 5 min denaturation cycle at 94˚C, 40 cycles of 30 sec at 94˚C (denaturation), 50 sec at 58˚C (annealing), 40 sec at 72˚C (amplification), and then a final 7 min cycle at 72˚C for amplification. The fragment amplified was 700 bp long.

The presence of the gene of interest *h6h* from pLAL21 was also verified by PCR. The primers used were GACAATGGAAGTGTGCAAAGAG and reverse AGCCCAATTTAAGCCCAAGT, and led to the amplification of a 330 bp fragment corresponding to exogenous *h6h* cDNA. As endogenous *h6h* DNA was also recognized by these primers, they were differentiated by the length of the amplified fragment (770 bp for endogenous *h6h*, as the fragment contains an intron). The PCR cycle was a 5 min denaturation cycle at 94˚C, 40 cycles of 30 sec at 94˚C (denaturation), 50 sec at 56˚C (annealing), 50 sec at 72˚C (amplification), and then a final 7 min cycle at 72˚C for amplification.

STATISTICAL ANALYSIS

The statistics applied to the biochemical data employed Student's t-test (p=0.05).

RESULTS

IMPROVEMENT OF GROWTH CHARACTERISTICS

In *Datura innoxia* hairy roots, tropane alkaloid production is highest in the late stationary phase after 15 days of culture (Boitel et al., 1996). Improvement of the biomass produced during the growth phase is one way to attempt to improve tropane alkaloid production in those hairy root cultures. The main function of the bioreactor is to produce biomass in controlled conditions (temperature, pH, dissolved oxygen).

The results of studying culture growth in bioreactors showed an uninterrupted increase of biomass for 20 days, during which the roots gradually absorbed the culture medium and filled the entire liquid volume of the reactor (Fig. 3). At the end of the batch, the apparent density of produced roots seemed to differ depending on the position of the roots inside the reactor . The root matrix could be divided roughly into three zones

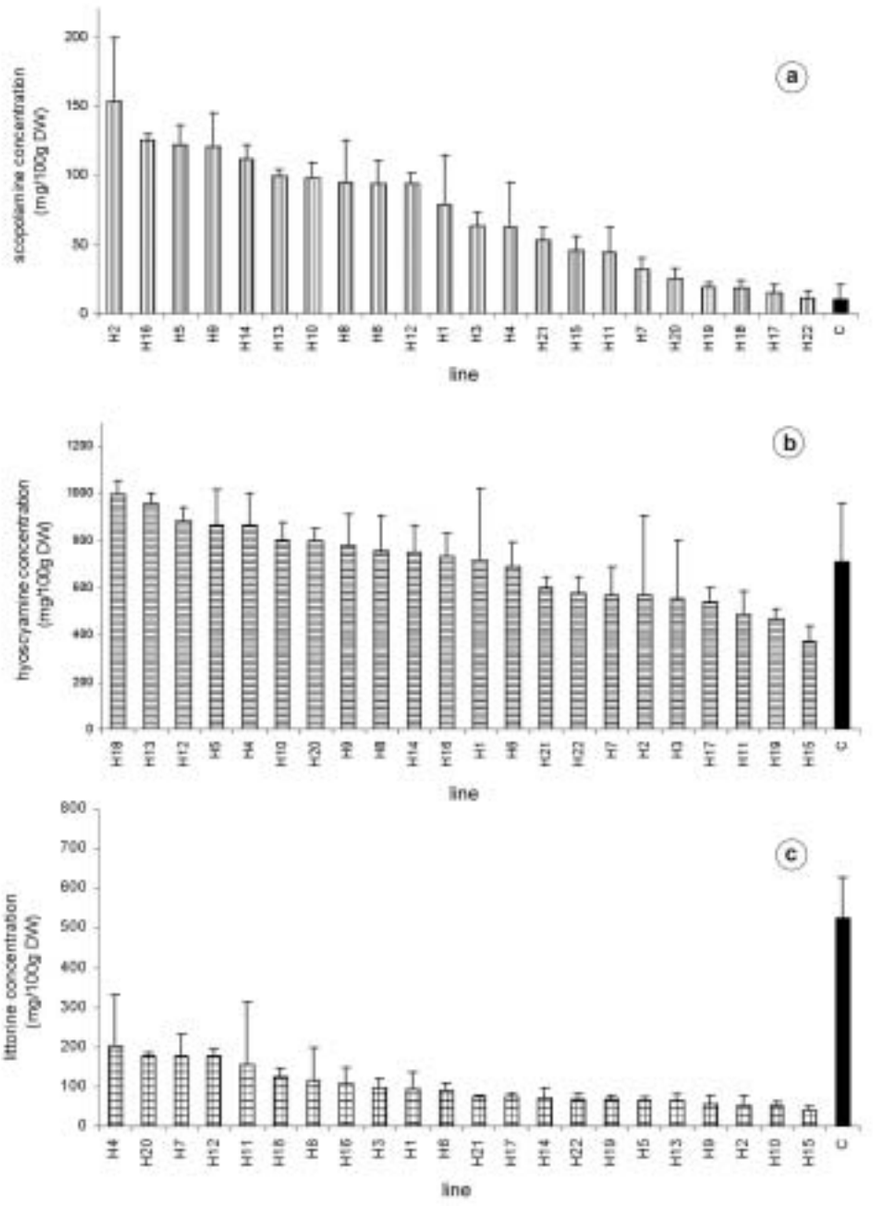

Fig. 5. Scopolamine (**a**), hyoscyamine (**b**) and littorine (**c**) content (mg/100g DW) in transformed lines directly after transformation. Black bar – control. The alkaloids of each line were analyzed for 7 different subcultures after 21 days of culture.

(Fig. 4): the upper part, consisting of old, densely entangled roots, occupying nearly 50% of the volume; the intermediate part, with only ~35%; and the bottom of the reactor, with narrow young roots occupying only 17% of the volume. The final results of 20 days of culture and the general equation of biomass kinetics are summarized in Table 1. Under the studied conditions, oxygen was provided in large excess, implying that the growth rate ($\mu = \ln 2/T_{Double}$) of roots was not limited by oxygen transfer in the culture medium. However, the composition of the culture medium needs to be optimized, especially regarding the type and concentration of carbon and phosphate sources. Scopolamine is the most valuable tropane alkaloid. In our hairy root clone of *Datura innoxia*, the hyoscyamine/scopolamine proportion is unfavorable. Cul-

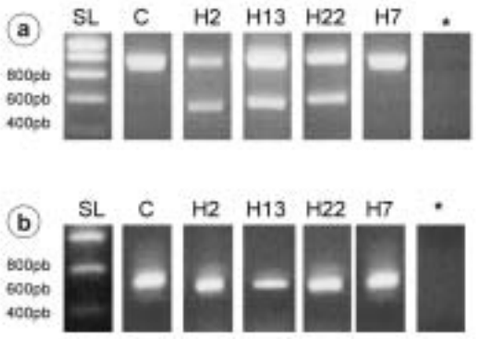

Fig. 6. Molecular analyses after transformation: PCR profiles in different lines and control (C). SL – Smart Ladder® molecular weight marker; * – negative control of PCR (without DNA). (**a**) Lower band: presence of exogenous *h6h* gene (330 bp); upper band: endogenous *h6h* gene (770 bp), (**b**) Presence of *rolA* gene (700 bp) confirming the hairy root phenotype in *h6h* lines.

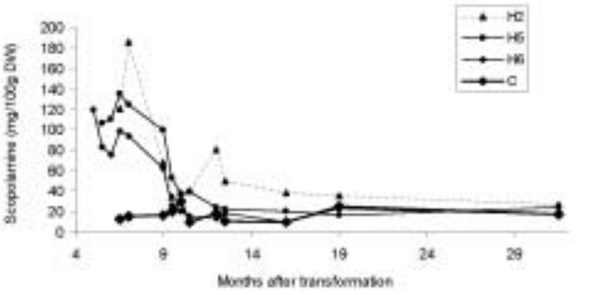

Fig. 7. Scopolamine concentrations during culture of some overproducing lines and control (C).

ture in a bioreactor leads to increased production of both but does not change the proportion of alkaloids. Only genetic engineering could resolve this problem.

GENETIC ENGINEERING OF TROPANE ALKALOIDS

In spite of advances in optimization of the growth conditions for genera used in alkaloid production, the yields remain too low to be economic (<0.05% DW scopolamine). Supplying labelled precursors in the culture medium in *Datura innoxia* hairy root cultures permits the limiting step to be identified (Lanoue et al., 2002). The last steps leading to scopolamine production seem to be rate-limiting.

Transformation with the *h6h* gene was done in different *Datura innoxia* plants, and the impact on tropane alkaloid production levels was studied in the obtained lines. As in other genera, introduction of the *h6h* gene in *Datura innoxia* hairy root lines led to the emergence of very different characters of morphology, growth, and alkaloid production. As expected, the presence of the transgene stimulated scopolamine production in most cases. Overaccumulation of scopolamine was observed in 61% of the transformed lines (H1-H22) (Fig. 5a). In the most productive line, the scopolamine level reached 0.15% of DW (control <0.05% DW).

Among the numerous alkaloids of the pathway (Fig. 1), we studied mainly hyoscyamine and littorine, as precursors of scopolamine. Whereas hyoscyamine seemed to be accumulated with little decrease compared to the control (Fig. 5b), the accumulation of littorine was drastically altered in most of the transformed lines (Fig. 5c). The lowest level of littorine was 5% of the control level. Moreover, the decrease of littorine content seemed not to be directly related to scopolamine overproduction and *h6h* gene insertion. Indeed, nearly all the lines were affected by the decrease of littorine in spite of the differences in molecular profiles (Fig. 6). For example, the H7 line accumulated very little littorine; the exogenous *h6h* gene could not be detected in this line.

The growth of those new hairy root lines was also markedly affected: their dry weight was 50% of the control weight after 21 days of liquid culture in the same conditions. No direct relation was found between growth ability and scopolamine production, despite the toxicity of this molecule. Thus, this lower growth could be due to insufficient medium supplementation for the lines' growth requirements. As mentioned before, the growth medium should be optimized for each line to permit better growth and consequently better alkaloid production.

ALKALOID STABILITY

In this work we analyzed alkaloid production in different lines during three years of subcultures after transformation. Unlike Jouhikainen et al. (1999), who observed stability of production during two and a half years, the scopolamine content in the *Datura innoxia* hairy roots we studied decreased to the control level after one year of subcultures. The recent results we obtained three years after transformation show levels of scopolamine stabilized at very low levels similar to the control (Fig. 7).

Only slight modifications of hyoscyamine production were observed, and only in the most overproducing lines. A slight increase of littorine content was found, but the concentrations were still very low compared to the control. Identification of the presence of the *h6h* gene (Polymerase Chain Reaction *h6h* profiles) in the different studied clones showed the same result as two years ago. Moreover, preliminary results on the transcription level of the *h6h* gene in the overproducing lines indicate that this gene is still overtranscribed in the transformed lines compared to the control. Thus,

the decrease of scopolamine accumulation observed in those lines was not due to molecular modification of the exogenous *h6h* gene.

DISCUSSION

The results presented here highlight the importance of taking into account medium composition and metabolite production together to produce useful levels of yield. However, it seems that *Datura innoxia* hairy root cultures are not able to stably overaccumulate scopolamine, as a decrease to the control level was observed after one year of subcultures. As the decrease was continuous to the control level and nonreversible over three years, we conclude that it was not due to a seasonal production cycle (Sporer et al., 1993). The experimental results show that this evolution of scopolamine content may be due to metabolic regulation: the high content of scopolamine accumulated in hairy roots, which have no storage structure or function, seems to be an enzymatic regulation signal. This suggestion is now under investigation.

In the scopolamine-rich plant *Duboisia*, Palazon et al. (2003) succeeded in obtaining *Duboisia* lines overproducing scopolamine (2.5 g/100g DW). They concluded that adding H6H activity in this plant may lead to better conversion of hyoscyamine to scopolamine, and consequently that hyoscyamine may be a feedback regulation signal in alkaloid accumulation. In the transformed lines they obtained, this inhibition was removed by the production of scopolamine. Thus each genus seems to possess its own regulation pathway, reflecting the mother plant's profile.

Sato et al. (2001) introduced the *pmt* gene of tobacco under the control of the 35S promoter in *Atropa belladonna*. The enzyme PMT (putrescine N-methyltransferase), responsible for reactions in the first steps of the tropane alkaloid pathway, is located at a key branch-point. This enzyme is very sensitive to external stress, which brings a decrease in its activity, leading to increased production of polyamines. Introduction of the gene did not lead to overaccumulation of tropane alkaloids or of any derivatives such as calystegines or nortropane alkaloids, in spite of N-methylputrescine accumulation (Rothe et al., 2003). The same results have been observed in *Duboisia* hairy root cultures transformed with the same construction (Moyano et al., 2002). Moreover, transformation of both *Datura metel* and *Hyoscyamus muticus* by the *pmt* gene led to accumulation of hyoscyamine (Moyano et al., 2003).

All these results show that the tropane alkaloid pathway is strongly regulated, and that the introduction of an exogenous gene coding for an enzyme of this pathway does not always lead to an increase in scopolamine production levels. Our experience with the molecular, biochemical and bioengineering aspects of this process confirms our initial hypothesis that transformed roots in axenic culture present a good model system for studying the regulation aspects of secondary metabolism in *Datura innoxia*.

ACKNOWLEDGEMENTS

We are greatly saddened at the death of our colleague, Professor Lesław Przywara, who always showed great interest in this biotechnology approach to plant physiology. We are grateful to Professor Elżbieta Kuta for giving us the opportunity to contribute to this special issue dedicated to his memory.

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