SHORT COMMUNICATION

Biotransformation of hyoscyamine into scopolamine in transgenic tobacco cell cultures

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Summary

Hyoscyamine-6β-hydroxylase (H6H) catalyses the conversion of hyoscyamine into its epoxide scopolamine, a compound with a higher added value in the pharmaceutical market than hyoscyamine. We report the establishment of tobacco cell cultures carrying the *Hyoscyamus muticus h6h* gene under the control of the promoter CAMV 35S. The cell cultures were derived from hairy roots obtained via genetically modified *Agrobacterium rhizogenes* carrying the pRi and pLAL21 plasmids. The cultures were fed with hyoscyamine, and 4 weeks later the amount of scopolamine produced was quantified by HPLC. The transgenic cell suspension cultures showed a considerable capacity for the bioconversion of hyoscyamine into scopolamine, and released it to the culture medium. Although the scale-up from shake-flask to bioreactor culture usually results in reduced productivities, our transgenic cells grown in a 5-L turbine stirred tank reactor in a batch mode significantly increased the scopolamine accumulation.

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Introduction

In the last decade, several investigators have used metabolic engineering of pharmaceutically important tropane alkaloids in order to improve the conversion of hyoscyamine to the more valuable...
subcultures over 4 weeks, the hairy roots were described previously by Häkkinen et al. (2005). It transgene from 11.5 root line were transferred to MS medium added to fragments of 100 mg fresh weight of the H6H14 tissues and obtain a cell suspension culture, lines obtained. In order to dedifferentiate root hyoscyamine into scopolamine out of all the root expression and the greatest capacity to convert was selected because it showed a high transgene expressed by Palazón et al. (1995). After 3

**Material and methods**

The tobacco root line H6H14, carrying the 35S-h6h transgene from Hyoscyamus, was obtained as described previously by Häkkinnen et al. (2005). It was selected because it showed a high transgene expression and the greatest capacity to convert hyoscyamine into scopolamine out of all the root lines obtained. In order to dedifferentiate root tissues and obtain a cell suspension culture, fragments of 100 mg fresh weight of the H6H14 root line were transferred to MS medium added to 11.5 μM IAA and 1 μM kinetin as previously described by Palazón et al. (1995). After 3–4 subcultures over 4 weeks, the hairy roots were transformed into friable callus masses from which cell suspension cultures were established. Callus pieces (∼100 mg) were inoculated into a 100 mL flask (Sigma) containing 40 mL MS liquid medium supplemented with the aforementioned hormonal concentration and capped with a Magenta B-Cap (Sigma) and then placed in a rotatory shaker at 100 rpm in the dark at 25°C. In order to obtain a fine cell suspension, cell cultures were passed through a stainless steel sieve (0.76 × 0.76 mm) every 2 weeks for 6 months and maintained in the same culture conditions. Transgenic tobacco cell cultures were entrapped in alginate beads as described by Gillet et al. (2000) and cultivated as for free cell cultures.

Free and immobilized cell cultures were fed with hyoscyamine added to the culture medium. About 0.5 ± 0.02 g of transgenic cells were grown in 40 mL liquid MS medium supplemented with 11.5 μM IAA and 1 μM kinetin. The hyoscyamine hydrochloride (Sigma) was dissolved in water and the stock was added to the final concentration of 100 or 200 mg/L. In control samples, only water was added. The cells were grown as described above for a culture period of 28 days. Alkaloid levels were determined both in the cells and the culture medium at the end of the culture period.

The growth was measured after 28 days of culture and expressed as Growth Index (GI) (final and initial fresh weight ratio). In the case of immobilized cell cultures, the beads were first disrupted as described by Bentebibel et al. (2005). The tropane alkaloids scopolamine and hyoscyamine were extracted and analysed from lyophilised cells and medium as described by Plank and Wagner (1986).

For bioreactor cultures, a commercially available 5-L turbine stirred tank bioreactor (Applikon Dependable Instruments, Schiedam, The Netherlands) (Cusido et al., 2002) was used. The culture was aerated through a sintered steel sparger. The flow was set at 0.8 L/min at the beginning of the experiment and then gradually increased up to 1.5 L/min and maintained at this level with a mass flow control system until the end of the culture period (Brooks, Veenendaal, The Netherlands). The working volume was maintained at 3 L culture medium. The inoculum was 80 g/L of free cells fresh weight, and the cell cultures were kept at 25°C in the dark for a culture period of 4 weeks.

**Results and discussion**

As previously reported (Häkkinen et al., 2005), we obtained several transgenic tobacco root lines
carrying the 35S-h6h transgene. Three of these root lines showed a high capacity to bioconvert the added hyoscymine into scopolamine. Depending on the root line, the scopolamine production ranged from 5.7 to 64.1 mg/L, the best results being achieved with the root line H6H14. In order to scale-up the process, this root line was now dedifferentiated and a fine cell suspension was obtained. The biomass production, measured as GI at the end of the culture (GI = 13.6) was very similar in the hairy root cultures (GI = 14.0) (Häkkinen et al., 2005). Adding hyoscymine to the medium of transformed cell cultures did not significantly affect their growth capacity (Table 1). Goossens et al. (2003) have also reported that the tobacco cell line BY-2 is able to tolerate hyoscymine even up to 3 g/L.

Similarly to the original H6H14 hairy root line from which our cell suspension culture was derived, the transgenic cell cultures showed a moderate capacity to bioconvert hyoscymine into scopolamine, when the cell suspensions were fed with 100 or 200 mg/L hyoscymine (Table 1). The total amount of scopolamine achieved at the end of the culture period was 16 and 21.6 mg/L, respectively, which is approximately 38% of that in the transgenic roots. There was no lack of substrate for bioconversion, and the best ratio of hyoscymine bioconversion into scopolamine (16.0%) had already been obtained by feeding the culture with 100 mg/L hyoscymine. Nevertheless, the capacity of transgenic cell cultures to secrete the produced scopolamine into the culture medium (secretion rate) was lower (18–20%) than that of the root line (56%).

Cell suspensions were immobilized in 1.25% of alginate and 0.8 M of CaCl₂, which were the exceptional conditions found to be optimal by Gillet et al. (2000) for the production and excretion of scopolamine by immobilized N. tacabum cells. After a culture period of 28 days, the growth (measured as GI) was over 10 times lower than in free cell suspensions, indicating that the immobilized cells remained in a stationary growth state throughout the culture period. The total scopolamine levels (cell-associated+extracellular) were dramatically reduced by cell immobilization (Table 1). When the culture medium was supplemented with 100 and 200 mg/L hyoscymine, the scopolamine contents were only 2.2 and 2.5 mg/L, respectively. That is, they were 7.3- and 8.6-fold lower than those obtained in the free cell cultures. Such low scopolamine levels observed in the immobilized cultures could be due to problems in the diffusion of hyoscymine, since the take-up of this precursor from the medium by immobilized cells was low, the percentage of absorption being only about 28% of the alkaloid supplied to the culture medium (Table 1). In contrast, the entrapment conditions were favourable for scopolamine excretion. The secretion rate from the producer cells to the medium was 74.0% and 80.7% in the cultures supplemented, respectively, with 100 and 200 mg/L of hyoscymine. The excretion percentages obtained with our immobilized cell cultures were 4- and 3.9-fold higher than those obtained in free cell suspensions, and their capacity to release scopolamine to the medium was also higher than that of the hairy root line H6H14.

With the aim of carrying out the envisaged scale-up of the process, 80 g/L fresh weight of the cells was inoculated in 3 L of medium in a 5 L turbine stirred tank bioreactor (Cusidó et al., 2002). The culture medium was supplemented with 200 mg/L

<table>
<thead>
<tr>
<th>Cell culture</th>
<th>Scale</th>
<th>Growth Index</th>
<th>Hyoscymine*</th>
<th>Scopolamine</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Absorption (%)</td>
<td>Total (mg/L)</td>
</tr>
<tr>
<td><strong>Free cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MS</td>
<td>Shake flask</td>
<td>13.6</td>
<td>—</td>
<td>—</td>
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<tr>
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<td>38</td>
<td>16.0 ± 0.4</td>
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<tr>
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<td>31.5</td>
<td>21.6 ± 1.1</td>
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<tr>
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<td>Bioreactor</td>
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<td>35.5 ± 0.8</td>
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<td><strong>Immobilized cells</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MS</td>
<td>Shake flask</td>
<td>1.1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>MS+H100</td>
<td>Shake flask</td>
<td>1.1</td>
<td>28</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>MS+H200</td>
<td>Shake flask</td>
<td>1.1</td>
<td>27</td>
<td>2.5 ± 0.1</td>
</tr>
</tbody>
</table>

Each value is the average of three replicates ± SD.

*Bioconversion of hyoscymine to scopolamine.

Table 1. Growth Index and scopolamine (medium and cell-associated) contents in H6H14 free and immobilized cell cultures at the end of the culture period (28 days) in liquid medium supplemented with 100 mg/L (H100) or 200 mg/L (H200) hyoscymine.
hyoscyamine, since in our small-scale assay we had obtained the highest levels of scopolamine using this concentration of the precursor. Although the growth decreased (GI = 4.8) substantially, the scopolamine level was significantly higher than that obtained in shake flasks. The total content (cell-associated+extracellular) of scopolamine was 35.5 mg/L, which was 1.6 times higher than that obtained in small-scale cultures. In this case almost 18% of the hyoscyamine added to the medium was transformed into scopolamine, which represented an increase of 65% with respect to the same alkaloid obtained by bioconversion in shake flasks.

These results suggest that plant cells which do not produce secondary compounds of interest are able to do so after bioconversion of a suitable precursor, if they overexpress the gene coding for the key enzyme involved in the biosynthesis of such compounds. We have shown that *N. tabacum* free cell suspensions, which normally do not produce scopolamine, are able to produce this tropane alkaloid after overexpressing the gene h6h, which codes for the last part of the scopolamine biosynthesis, and after being fed with the immediate precursor hyoscyamine.

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