EFFECTS OF ELICITORS ON TROPANE ALKALOID PRODUCTION AND RELEASE IN TRANSFORMED ROOT CULTURES OF BRUGMANSIA CANDIDA

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Abstract

Scopolamine and hyoscyamine are tropane alkaloids employed in medicine as antispasmodics and for the treatment of motion sickness. Both compounds are extracted from plants that belong to several species of Solanaceae because their chemical synthesis is complicated and expensive. Obtaining these compounds through in vitro cultures is a promising alternative. In this research, transformed (hairy) roots of Brugmansia candida have been used. Different clones were obtained through infection with the soil pathogen Agrobacterium rhizogenes, and transformation was confirmed by PCR. Almost all the clones dedifferentiated, and in some cases this could be reverted using auxins or modifying the culture media. In order to increase alkaloid productivity, hairy roots with normal morphology were treated with a biotic elicitor (hemicellulase) and a stress agent (CuSO₄). Hemicellulase had a positive effect on production of both alkaloids (100-200%). When CuSO₄ was used, there was a dramatic increase in the release of both alkaloids (500%) into the medium.

1. Introduction

The tropane alkaloids scopolamine and hyoscyamine are anticholinergic agents employed in medicine as antispasmodics and for the treatment of motion sickness. Both compounds are extracted from plants that belong to several species of Solanaceae because their chemical synthesis is complicated and expensive (Yamada, 1994). In vitro culture techniques are an interesting alternative to obtain both substances, since they would guarantee a stable and uniform year-round supply. These metabolites are only synthesized in organized tissues and the in vitro culture of cells derived from different tropane alkaloid producing species has therefore been unsuccessful. Because they are biosynthesized in the roots of the plant, the culture of roots is the most appropriate in vitro system to obtain them.

Hairy roots of B. candida (Solanaceae), a South American plant which produces scopolamine and hyoscyamine, were used in this research. Different clones of hairy roots were obtained through infection of explants with the soil pathogen A. rhizogenes. Transformed root cultures present advantageous characteristics compared with normal roots: they are genetically stable, achieve high growth rates in hormone-free media and their pattern of alkaloid production is similar to that observed in the parent plants (Flores, 1992).

To increase the productivity of in vitro plant culture systems, many strategies have been assayed. Elicitation has proven to be one of the most successful ones. Biotic
elicitors (macromolecules that originate either from the plant or a plant pathogen) and stress agents (heavy metal salts, UV radiation, etc.) induce in plants the biosynthesis of defense compounds. In an attempt to increase tropane alkaloid production, hairy root cultures of \textit{B. candida} were exposed to a both a biotic elicitor (hemicellulase) and a stress agent (CuSO\textsubscript{4}).

2. Materials and Methods

2.1. Establishment of axenic root cultures

Transformed roots were obtained by infection of sterile seedlings of \textit{B. candida} with \textit{A. rhizogenes} LBA 9402, constituting each root tip a separate clone. They were transferred to hormone-free Murashige and Skoog (1962) medium plus the RT vitamin complex described by Khanna and Staba (1969) (MSRT), with the addition of 30 g/l sucrose. Axenic cultures were obtained employing ceftizoxime. The transformation of clones 7A and 12B were confirmed according to the procedure described by Hamill \textit{et al.} (1991).

2.2. Modification of the culture medium

The medium described by Gamborg (B5) (Gamborg \textit{et al.}, 1969), but with half concentration of mineral salts and vitamins (B5\textsubscript{1/2}) was tested. The sucrose concentration was also reduced in half (from 30 g/l to 15 g/l).

2.3. Addition of anti-auxin

The anti-auxin 2(p-chlorophenoxi)-2-methyl propionic acid (PCMP) was added to B5\textsubscript{1/2} medium, supplemented with 30 g/l sucrose, at a concentration of 0.10 mg/l.

2.4. Maintenance of hairy root cultures

The roots that were used in the experiments with elicitors were maintained on hormone-free B5\textsubscript{1/2} medium, supplemented with 15 g/l sucrose. They were subcultured every 15-20 days and incubated at 24 ± 2°C, in gyratory shakers at 100 rpm. A 16-h photoperiod was applied using cool white fluorescent lamps at a light intensity of approximately 1.8 \text{wm}^{-2}.

2.5. Assays with biotic elicitors and stress agents

Approximately 50-100 mg (fresh weight:FW) of hairy roots (clone 7A) were inoculated in 25 ml of B5\textsubscript{1/2} (with 15 g/l sucrose) contained in 125 ml Erlenmeyers. The cultures were incubated as described above. The elicitors were added on the 18th day of culture and samples were taken after 24, 48 and 72 hours. Growth (fresh weight: FW), scopoline and hyoscyamine accumulation in the roots and release into the medium were determined.

1) \textit{Hemicellulase}: A stock solution was prepared by dissolving hemicellulase (from \textit{Aspergillus niger}) in distilled water. Two final concentrations were employed: 60.00 μg/ml and 120.00 μg/ml (corresponding to 0.02 U/flask and 0.04 U/flask). The solution was filter sterilized prior to its addition.

2) CuSO\textsubscript{4}: The salt, prior sterilization by autoclaving at 120°C and 1 atm during 20 min., was added in two final concentrations: 1.00 and 2.00 mM.
2.6. Analytical Methods

FW was determined by separating the root tissue from the medium by vacuum filtration. Alkaloid extraction was carried out as described by Parr et al. (1990). Hyoscyamine and scopolamine were analyzed by HPLC, according to the method described by Mano et al. (1986).

3. Results

3.1. Selection of the clones

Clones 7A and 12B were selected in view of their high alkaloid productivity (data not shown). Their transformation was confirmed according to the procedure described above. Both clones exhibited a tendency towards dedifferentiation in liquid MSRT medium. The process typically began with the appearance of small calli, which gradually proliferated, finally forming a compact callus. Dedifferentiation resulted in a decline in alkaloid production (Pitta-Alvarez and Giulietti, 1995).

3.2. Re-differentiation strategies

The changes in the culture medium described in Materials and Methods, which reduced the osmotic stress through a decrease in salt, vitamin and sucrose concentrations, and the use of anti-auxins were both successful in reverting the dedifferentiation process in clone 7A, and the reappearance of normal and stable root morphology could be observed. Clone 12B, however, did not respond to any of the above mentioned treatments.

3.3. Elicitation with hemicellulase

In Table 1, it can be observed that hemicellulase, in both concentrations tested and after 24 h, had a positive effect on scopolamine and, especially, hyoscyamine accumulation in the roots. This effect was also seen, although to a lesser extent, after 48 h. Release of alkaloids into the medium was enhanced at 48 h, but only for scopolamine.

3.4. Elicitation with CuSO₄

Table 1 also shows that the main effect of CuSO₄ was on the release of alkaloids into the medium, particularly after 24 and 48 hs. Accumulation in the roots descended dramatically and, after 72 hs, growth and alkaloid production were negatively affected.

4. Discussion

The reversal of the dedifferentiation process in clone 7A by modifying the culture medium, suggests that these hairy roots are particularly susceptible to osmotic stress. Furthermore, the successful use of anti-auxins suggests that they also exhibit an exceptionally high sensitivity toward auxins.

With respect to the results observed when the roots were treated with CuSO₄, the increase in the release of both alkaloids simultaneous to a decrease in their accumulation in the roots, could be indicating a possible effect of this salt on membrane permeability.
The negative effects on growth and alkaloid production observed after 72 hs are probably due to the toxicity of the copper salt.

The full nature of elicitor-induced biochemical changes is not wholly understood. Tropane alkaloids are constitutively produced: they are synthesized at a steadily low rate and accumulate in specific tissue. Frequently, compounds in this category show no increase in biosynthesis in response to elicitation. However, in this research it has been shown that hairy roots of *B. candida* can respond to biotic elicitors and stress agents.

5. References

Table 1 - Effect of different elicitor treatments on accumulation and release of scopolamine and hyoscyamine in transformed root cultures of *Brugmansia candida*. The results are expressed as the percentage increase or decrease compared to the corresponding control.

<table>
<thead>
<tr>
<th>Hours</th>
<th>Treatment</th>
<th>FW (%)</th>
<th>SR (%)</th>
<th>Sm (%)</th>
<th>Hr (%)</th>
<th>Hm (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>Hemicellulase 60.00 µg/ml</td>
<td>44</td>
<td>150</td>
<td>-10</td>
<td>200</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Hemicellulase 120.00 µg/ml</td>
<td>56</td>
<td>80</td>
<td>-96</td>
<td>340</td>
<td>-80</td>
</tr>
<tr>
<td></td>
<td>CuSO₄ 1.00 mM</td>
<td>-5</td>
<td>0</td>
<td>500</td>
<td>-65</td>
<td>700</td>
</tr>
<tr>
<td></td>
<td>CuSO₄ 2.00 mM</td>
<td>-45</td>
<td>-5</td>
<td>100</td>
<td>-99</td>
<td>100</td>
</tr>
<tr>
<td>48</td>
<td>Hemicellulase 60.00 µg/ml</td>
<td>40</td>
<td>30</td>
<td>200</td>
<td>40</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Hemicellulase 120.00 µg/ml</td>
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<td>30</td>
<td>200</td>
<td>70</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>CuSO₄ 1.00 mM</td>
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<td>-83</td>
<td>900</td>
<td>-70</td>
<td>900</td>
</tr>
<tr>
<td></td>
<td>CuSO₄ 2.00 mM</td>
<td>-10</td>
<td>-90</td>
<td>900</td>
<td>-97</td>
<td>1100</td>
</tr>
</tbody>
</table>

FW: fresh weight; Sr: scopolamine accumulated in the roots; Sm: scopolamine released into the medium; Hr: hyoscyamine accumulated in the roots; Hm: hyoscyamine released into the medium.

ND: Scopolamine or hyoscyamine not detected in the medium.

Each value represents the mean of three independent determinations that typically differed in 10%.