*Biotechnology Letters* **22:** 1653–1656, 2000.



# Scopolamine and hyoscyamine production by hairy root cultures of *Brugmansia candida*: influence of calcium chloride, hemicellulase and theophylline

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Received 17 July 2000; Revisions requested 26 July 2000; Revisions received 22 August 2000; Accepted 23 August 2000

*Key words: Brugmansia candida*, hairy root cultures, hyoscyamine, scopolamine

**Abstract**

$CaCl_2$ (50 mM) and hemicellulase (0.5 U $mg^{-1}$) increased the intracellular accumulation (60–250%), release (60–200%) and production (45–200%) of hyoscyamine and scopolamine in hairy roots of *Brugmansia candida*. Theophylline (0.25 mM), alone or in combination with hemicellulase, was ineffective in increasing hyoscyamine and scopolamine production.

## Introduction

Hairy root cultures of *Brugmansia candida* (Solanaceae) have been established to produce scopolamine and hyoscyamine (Pitta-Alvarez & Giulietti 1995). One of the main drawbacks of *in vitro* plant cell and organ cultures for the production of secondary metabolites is their low yields (Buitelaar & Tramper 1992). Elicitation, which stimulates defense responses in plants, is a frequent approach to improve them (Mukundan & Hjortso 1990, Ramakrishna *et al.* 1993, Chang *et al.* 1998). In studies of signal transduction pathways in plant defense mechanisms, $Ca^{2+}$ has been proposed as a second messenger (Nishi 1994). The role of cAMP, however, is not clear (Walden 1998), but cAMP participates in diverse physiological events within plant cells (Assmann 1995). Theophylline is an inhibitor of cAMP phosphodiesterase, increasing the intracellular concentration of cAMP. Nishi (1994) has reported that phytoalexin biosynthesis can be stimulated with theophylline alone. Therefore, theophylline could be used to enhance the response of plant cell cultures to elicitors. In this paper, the effect of $CaCl_2$ (abiotic elicitor and second messenger), hemicellulase (biotic elicitor) and theophylline on production and release of hyoscyamine and scopolamine in hairy roots *B. candida* is reported. In addition, the influence of the growth phase in elicitation response is also studied.

## Materials and methods

### *Initiation, maintenance and elicitation of hairy root cultures*

Hairy root cultures of *B. candida* were established and maintained as described in Pitta-Alvarez & Giulietti (1995). In elicitation experiments, 50–100 mg 15-day-old root tips were transferred to 25 ml of hormone-free Gamborg medium (Gamborg *et al.* 1968) with half-concentration of salts and vitamins ($B5_{1/2}$),supplemented with 30 g sucrose $l^{-1}$, contained in 125-ml Erlenmeyer flasks. They were incubated at 24 ± 2 °C, in gyratory shakers at 100 rpm with a 16-h photoperiod using cool, white fluorescent lamps at a light intensity of approximately 1.8 $wm^{-2}$. The roots were exposed to the elicitors for periods of 24 and 48 h. Fresh weight (FW) and hyoscyamine and scopolamine in the roots and the medium were determined.

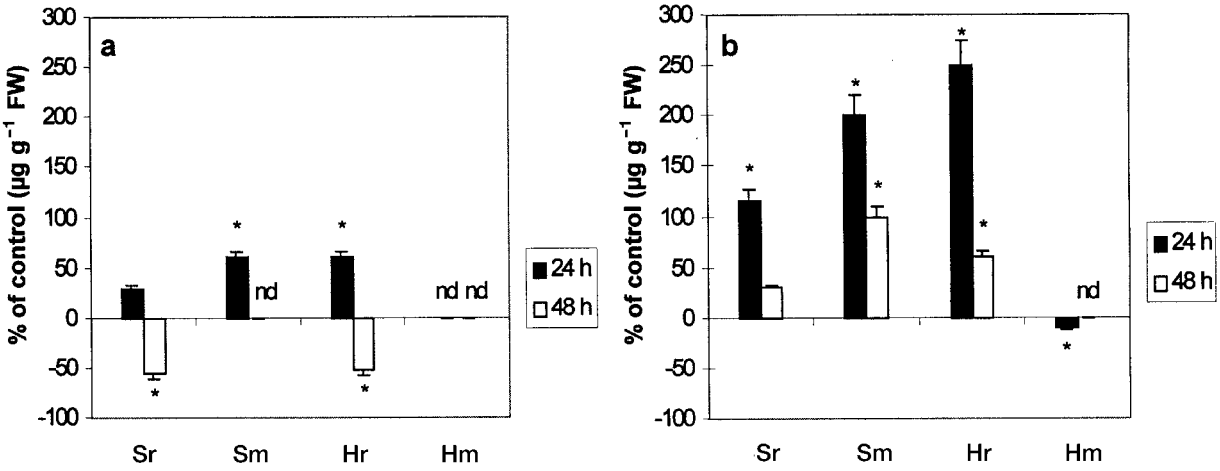


*Fig. 1.* Effect of (a) $CaCl_2$ 50 mM and (b) hemicellulase 100 $\mu$g ml$^{-1}$ on the accumulation and release of scopolamine and hyoscyamine in hairy root cultures of *B. candida*. Sr: scopolamine in roots, Hr: hyoscyamine in roots; Sm: scopolamine released into the medium; Hm: hyoscyamine released into the medium; nd: not detected. Control values (in $\mu$g g$^{-1}$ FW): Sr: 1200; Hr: 200; Sm: 40; Hm: 20. Absolute values (alkaloids in the biomass and in the medium expressed as percentages of the control absolute values): $CaCl_2$ 50 mM: Scopolamine: 30% (24 h) and −56% (48 h), Hyoscyamine: 45% (24 h) and −56% (48 h). Hemicellulase 100 $\mu$g ml$^{-1}$: Scopolamine: 117% (24 h) and 32% (48 h), Hyoscyamine: 226% (24 h) and −45% (48 h). Data marked with (*) are significantly different with respect to the corresponding control according to Tukey's test ($p = 0.05$).

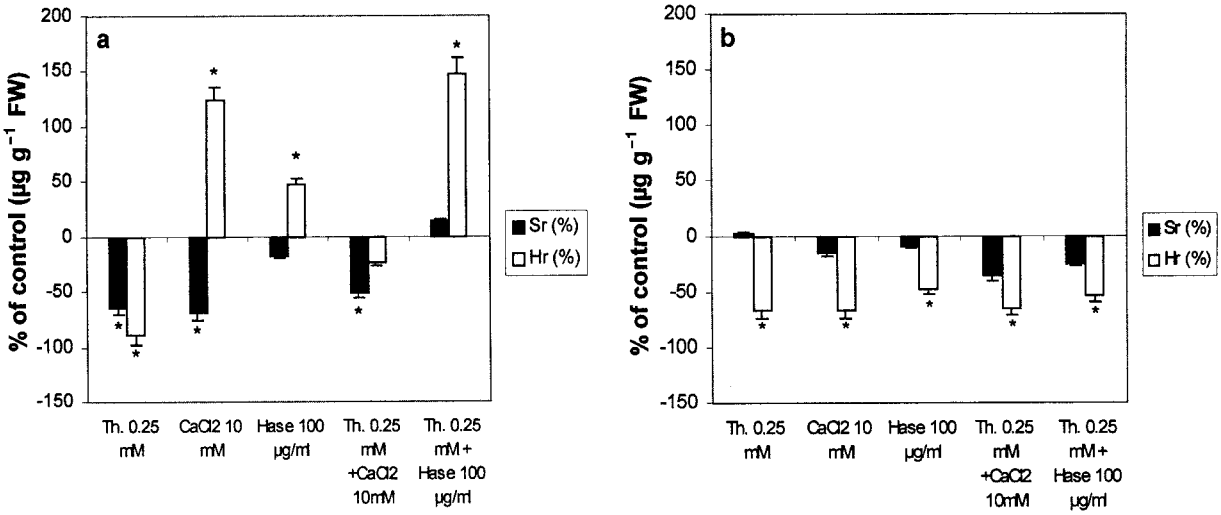


*Fig. 2.* Effect of theophylline 0.25 mM, $CaCl_2$ 10 mM and hemicellulase 100 $\mu$g ml$^{-1}$ on accumulation of scopolamine and hyoscyamine in hairy root cultures of *B. candida* after (a) 24 h and (b) 48 h. Abbreviations used in the figure: Theophylline: Th; Hemicellulase: Hase. Sr: scopolamine in roots, Hr: hyoscyamine in roots. Control values (in $\mu$g g$^{-1}$ FW): Sr: 210 (24 h), 75 (48 h); Hr: 37 (24 h), 210 (48 h). The absolute values are the same as Sr and Hr because no alkaloids were detected in the medium. Data marked with (*) are significantly different with respect to the corresponding control according to Tukey's test ($p = 0.05$).

### *Elicitors*

$CaCl_2$ was added to 18-day old cultures to give 50 mM. Hemicellulase (5 U mg$^{-1}$) (from *Aspergillus niger*) in distilled water, was sterilized by filtration and added to 18 day-old and 30 day-old root cultures to give 100 $\mu$g ml$^{-1}$. Aminophylline (the soluble form of theophylline) was filter-sterilized and added alone or with the other elicitors to give 0.25 mM.

### *Analytical methods*

FW, alkaloid extraction and scopolamine and hyoscyamine determination were carried out as described in Pitta-Alvarez & Giulietti (1995).

*Statistical analysis*

Significance of treatment effects were determined using analysis of variance (ANOVA). Variations among treatment means were analyzed using Tukey's procedure (Tukey 1953) ($p = 0.05$).

## Results and discussion

Figure 1a shows that $CaCl_2$ (50 mM) increased the intracellular concentration of both alkaloids and the release of scopolamine after 24 h. These results are in agreement with the ones obtained by Gontier *et al.* (1994) although they found that $Ca^{2+}$ increased the intracellular content of scopolamine and hyoscyamine in a suspension culture of *Datura innoxia* 10-fold. However they used a lower concentration of $Ca^{2+}$ (10 mM), and employed cell cultures with initially low concentrations of both metabolites. As can be seen in Figure 1b, hemicellulase stimulated significantly the intracellular accumulation of scopolamine and hyoscyamine after 24 h. Scopolamine release into the medium was promoted for the duration of the experiment, while hyoscyamine release was unaffected. The production of scopolamine increased with $CaCl_2$ after 24 h (30%), and with hemicellulase after 24 h (117%) and 48 h (32%). With respect to hyoscyamine, its production increased with $CaCl_2$ (45%) and hemicellulase (226%) after 24 h, and also with the latter after 48 h (45%). There were no significant changes in FW (data not shown).

Plant cells respond poorly to elicitors when the culture has started to accumulate inducible compounds, which is usually during the late exponential phase, and the procedure can even suppress biosynthesis (Brodelius & Pederson 1993). Therefore, most plant cell cultures respond only during the exponential growth phase. However, in the hairy roots used in this experiment, which grow following an exponential model, the tropane alkaloids accumulate during the exponential phase (Pitta-Alvarez & Giulietti 1995). Consequently, in an attempt to improve the response in this part of experiment, the roots were elicited in late exponential phase (30-day-old cultures), which is when production decreases. Furthermore, considering the results obtained by Gontier *et al.* (1994), the concentration of $CaCl_2$ tested was lowered to 10 mM.

Figure 2a shows that, after 24 h, theophylline produced a decrease of the production of hyoscyamine and scopolamine, while $CaCl_2$ and hemicellulase increased hyoscyamine (120%). $CaCl_2$ was unable to revert the negative effect of theophylline on hyoscyamine and scopolamine accumulation in roots, while the addition of hemicellulase to theophylline produced an increase in hyoscyamine of up to 148%. Figure 2b shows that, after 48 h of elicitation, there was a decrease of the accumulation of both alkaloids. Scopolamine and hyoscyamine were not detected in the medium. No significant changes in FW were observed (data not shown).

If we compare the results obtained in 18- and 30-day-old cultures, it is clear that the growth period in which the elicitor was added played a crucial role in the response. It is possible that in hairy roots of *B. candida* other secondary routes are more likely to be stimulated during late stationary phase, for example, sesquiterpenes, which are the phytoalexins in Solanaceae.

## Acknowledgements

This work was supported by Secretaría de Ciencia y Técnica de la Universidad de Buenos Aires (UBACyT), the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina, and the Fondo Nacional de Ciencia y Técnica (FONCYT).

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