

Effect of jasmonic acid and aluminum on production of tropane alkaloids in hairy root cultures of *Brugmansia candida*

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Hairy root cultures of *Brugmansia candida* (Solanaceae), a South American plant which produces scopolamine and hyoscyamine, were exposed to different elicitors (jasmonic acid (JA) and aluminum chloride ($AlCl_3$)) in order to increase their productivity and/or stimulate their liberation. Hairy roots of 19-day old cultures (exponential phase) were exposed to these elicitors for 24 and 48 hours. The effects on alkaloid accumulation and release into the medium were evaluated. JA was tested at 2.5 and 25 mg/ml. After 24 hours, JA promoted the release of hyoscyamine (~1200%) when the highest concentration was used. Therefore, the positive effects seen with JA could possibly be attributed in part to ethanol (EtOH), the solvent in which the acid was dissolved. At the lowest concentration tested, JA promoted an increase on scopolamine accumulation (30%) after 48 hours of exposure. When exposed to $AlCl_3$ for 48 hours and at concentrations of 25 and 250m M, scopolamine and hyoscyamine accumulation increased in the roots (43-83%). After 48 hours of treatment with the highest concentration of $AlCl_3$, release of scopolamine into the medium increased approximately 150%.

The tropane alkaloids scopolamine and hyoscyamine are anticholinergic agents employed in medicine. Since their

chemical synthesis is difficult and expensive, these compounds are still extracted from plants that belong to several species of the Solanaceae. Obtaining both substances through *in vitro* culture techniques is an interesting alternative, since it would guarantee a stable and uniform year-round supply, independent from seasonal variations of field-grown plants. Both alkaloids are synthesised in the roots of the plant; consequently the culture of normal and transformed roots is the most appropriate *in vitro* system to produce them.

The latter have several advantages, among them their high growth rate, genetic stability and production patterns that are similar to plants *in vivo* (Flores et al, 1999). One of the methods employed to enhance production is the use of elicitors. Exposure to biotic elicitors or to stress agents (abiotic elicitors) frequently induces the synthesis of secondary metabolites in plants (Benhamou, 1996).

In this work, hairy roots of *Brugmansia candida* (Solanaceae), a South American plant which produces both alkaloids, were exposed to different elicitors (JA and $AlCl_3$) in order to increase their productivity and/or their release. It has been previously established that treatments with exogenous jasmonates can increase the production of several classes of alkaloids in a range of plant species

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(Gundlach et al, 1992) particularly tropane alkaloids in *Datura stramonium* (Zabetakis et al, 1999). Aluminium, a toxic soil metal, has been described as an elicitor that up-regulates genes involved in the defence of tolerant species (Hamel et al, 1998).

Materials and Methods

Establishment and maintenance of hairy root cultures

Hairy roots were obtained by infection of sterile explants of *B. candida* with *Agrobacterium rhizogenes* LBA 9402, according to the procedure described in Pitta-Alvarez and Giulietti (1995). The roots were maintained in hormone-free Gamborg (Gamborg et al, 1969) (B5) medium containing half -concentration of mineral salts and vitamins (B5_{1/2}) and supplemented with 15 g/l sucrose. The roots were subcultured in the medium described above every 15-20 days and incubated at 24° ± 2° C, in shakers at 100 rpm with a 16-hour photoperiod using cool white fluorescent lamps at a light intensity of approximately 1.8 wm^{-2} .

Assays with elicitors

Approximately 150 mg (fresh weight: FW) of hairy roots were inoculated in 20 ml of B5_{1/2} medium supplemented with 15 g/l sucrose contained in 100 ml flasks. The cultures were incubated as described above.

JA was dissolved in EtOH and, after sterilising the solution by filtration, it was added in two final concentrations: 2.5 and 25 $\mu\text{g/ml}$. The AlCl_3 solution, after autoclaving at 120° C and 1atm during 20 minutes, was added in two final concentrations: 25 and 250 μM .

Hairy roots of *B. candida* in exponential phase (19 day-old cultures) were exposed to the elicitors for 24 and 48 hours. Growth (FW), scopolamine and hyoscyamine accumulation in the roots and release into the medium were determined.

Analytical methods

FW was determined by separating the root tissue from the medium by vacuum filtration. Alkaloid extraction from the roots and the media were carried out as described by Parr et al (1990). Hyoscyamine and scopolamine were analysed by HPLC, according to the method described by Mano et al (1986). The structures were confirmed by gas chromatography coupled with mass spectrometry, verifying also the absence of contaminants.

Statistical analysis

The treatments were carried out employing aleatory and factorial designs with three independent repetitions. Significance of treatment effects were determined using analysis of variance and comparison between the means of

the determinations. Due to the heterogeneity of the variances, the variables were transformed using Taylor's method employing the SAS program (SAS Institute Inc., 1989) licensed by INTA-Argentina (National Institute of Agronomic Technology).

Chemicals

Scopolamine hydrobromide, L-hyoscyamine hemisulfate, (-) JA, AlCl_3 , and all the media components were purchased from Sigma Chemical Co. (St. Louis, MO).

Results

Effects of JA and AlCl_3 on scopolamine and hyoscyamine accumulation on hairy root cultures of *B. candida*.

After 24 hours of elicitor treatment, there was a reduction in scopolamine and hyoscyamine contents (Figure 1a). However, after exposing the cultures to the elicitors for 48 hours (Figure 1b), the aluminium salt, at both concentrations tested, stimulated the production of scopolamine and hyoscyamine (43-83%). JA, at the lowest concentration tested, promoted an increase in the content of scopolamine (~30%), whilst the content of both alkaloids decreased when JA was used at its highest concentration. Treatment with EtOH had a negative effect on the accumulation of scopolamine.

Effects of JA and AlCl_3 on the release of scopolamine and hyoscyamine in hairy root cultures of *B. candida*

Elicitation with JA and AlCl_3 showed a positive effect on release of both alkaloids (Figures 1c and 1d). The most important effect was induced by JA at the highest concentration and after 24 hours of exposure, which promoted the release of hyoscyamine (~1200%) (Figure 1c). EtOH, the solvent in which JA was dissolved, also increased the release of hyoscyamine, but in a minor proportion (Figure 1c). Furthermore, according to the level of JA tested, the alkaloid profile was inverted, with scopolamine being prevalent when the lowest concentration was employed (Figures 1c and 1d). After 48 hours, the aluminium salt increased the release of scopolamine (~150%) only at its highest concentration (Figure 1d).

Discussion

Although chemical synthesis can be employed to replace plant exploitation, in the particular case of tropane alkaloids this procedure is expensive and time-consuming (Yamada et al, 1994). Plant cell and organ cultures present several advantages in comparison to field-grown plants, such as independence from variations induced by climatic and seasonal changes and losses due to plagues. Also, they allow the possibility of working in aseptic conditions. In

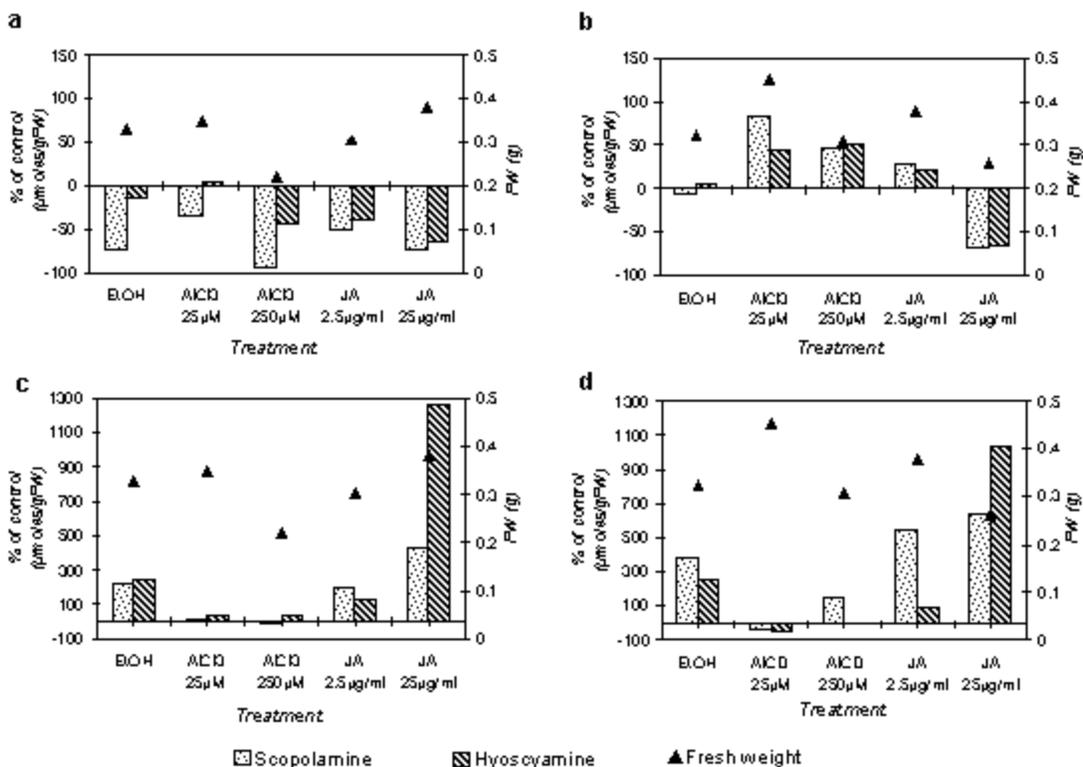


Figure 1. Effects of jasmonic acid (JA) and AlCl₃ in different concentrations and for different exposure times on the accumulation in roots and release of scopolamine and hyoscyamine in hairy roots cultures of *B. candida*

Results are expressed as FW (g) and percentages of increase or decrease of scopolamine and hyoscyamine with respect to the mean of corresponding control. Each value represents the mean of three independent determinations. The calculated variation coefficients are between 1.17 and 3.12%.

(a) Accumulation of tropane alkaloids (time of exposure: 24 h). Control values: tropane alkaloids (in µmoles/gFW): scopolamine(0.04); hyoscyamine(1.18). FW (g): 0.30.

(b) Accumulation of tropane alkaloids (time of exposure: 48 h). Control values: tropane alkaloids (in µmoles/gFW): scopolamine (0.08); hyoscyamine(0.79). FW (g): 0.33.

(c) Release of tropane alkaloids (time of exposure: 24 h). Control values: tropane alkaloids (in µmoles/gFW): scopolamine (0.18); hyoscyamine(0.12). FW (g): 0.30.

(d) Release of tropane alkaloids (time of exposure: 48 h) Control values: tropane alkaloids (in µmoles/gFW): scopolamine (0.04); hyoscyamine(0.19). FW (g): 0.33.

our research, hairy roots, and not undifferentiated cultures, were employed, because tropane alkaloids are synthesised in the roots of plants and their production is strictly correlated with a differentiated state (Flores et al, 1999).

Pitta-Alvarez (1998) and Pitta-Alvarez et al (2000) have reported that certain clones of hairy roots of *B. candida* are susceptible to elicitation, inducing the intracellular accumulation and release into the media of scopolamine and hyoscyamine.

This is particularly notable during exponential phase and for periods ranging from 24 to 72 hours. Secondary metabolism in plant cell cultures of different species could be elicited by exogenously supplied jasmonates in levels that varied from 0.1 to 100 m M (Gundlach et al, 1992, Blechert et al, 1995, Taguchi et al, 1998, Zabetakis et al, 1999).

However, in our experiments, the positive effects on release with JA could be attributed in part to EtOH,

although this particular interaction should be further examined.

Hamel et al (1998) found that most of the genes up-regulated by aluminium (in concentrations that ranged from 5 to 500 μ M) shared homologies with pathogenesis-related ones, suggesting that aluminium may act as an elicitor. This mode of action could also be involved in the hairy root system we employed, since there was an increase in the accumulation of both alkaloids and the release of scopolamine.

Elicitors, which affect positively the release of secondary metabolites (as was observed in our research work), represent a valuable biotechnological strategy. For example, in large-scale fermentation designed to obtain molecules from plant cultures, this approach could simplify and reduce the costs of downstream processes, and at the same time allow biomass reutilization.

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