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How Polyamine Synthesis Inhibitors and Cinnamic Acid Affect Tropane Alkaloid Production

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Abstract

Hairy roots of *Brugmansia candida* produce the tropane alkaloids scopolamine and hyoscyamine. In an attempt to divert the carbon flux from competing pathways and thus enhance productivity, the polyamine biosynthesis inhibitors cyclohexylamine (CHA) and methylglyoxal-bis-guanylhydrazone (MGBG) and the phenylalanine-ammonia-lyase inhibitor cinnamic acid were used. CHA decreased the specific productivity of both alkaloids but increased significantly the release of scopolamine (approx 500%) when it was added in the mid–exponential phase. However, when CHA was added for only 48 h during the exponential phase, the specific productivity of both alkaloids increased (approx 200%), favoring scopolamine. Treatment with MGBG was detrimental to growth but promoted release into the medium of both alkaloids. However, when it was added for 48 h during the exponential phase, MGBG increased the specific productivity (approx 200%) and release (250–1800%) of both alkaloids. Cinnamic acid alone also favored release but not specific productivity. When a combination of CHA or MGBG with cinnamic acid was used, the results obtained were approximately the same as with each polyamine biosynthesis inhibitor alone, although to a lesser extent. Regarding root morphology, CHA inhibited growth of primary roots and ramification. However, it had a positive effect on elongation of lateral roots.

Index Entries: *Brugmansia candida*; scopolamine; hyoscyamine; cyclohexylamine; methylglyoxal-bis-guanylhydrazone; *trans*-cinnamic acid; tropane alkaloids.

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Introduction

Brugmansia candida (syn. *Datura candida*) is a Solanaceae that produces the tropane alkaloids scopolamine and hyoscyamine *(1)*. These alkaloids are anticholinergic agents used extensively in medicine. Hairy roots of *B. candida* are efficient producers of both alkaloids *(2)*. To enhance the productivity of hairy root cultures in various tropane alkaloid–producing species, different approaches have been tested, ranging from elicitation *(3,4)* and addition of exogenous precursors *(5,6)* to overexpression of the genes involved in the biosynthetic pathway *(7–9)*. In particular, the results obtained when precursors are added exogenously are diverse and contradictory, because often the precursor is degraded or not taken up *(5,6)*. In the present work, a different approach was taken employing different enzyme inhibitors to attempt to divert the carbon flux from competing pathways and in turn increase endogenous precursors.

The polyamine putrescine (Put) is a molecule common to both primary and secondary metabolism in datura. It can be converted into other polyamines, such as spermidine (Spd) and spermine (Spm), and, together with them, participate in essential processes of primary metabolism related to plant growth and development *(10–12)*. However, Put can also enter the tropane alkaloid pathway through putrescine methyltransferase (PMT), the enzyme that catalyzes the first committed step in this synthesis (Fig. 1). In the present work, the polyamine synthesis inhibitors cyclohexylamine (CHA) and methylglyoxal-bis-guanylhydrazone (MGBG) were employed aiming to block the synthesis of Spd and / or Spm, with the hypothesis that Put could be diverted into the tropane alkaloid route. CHA is a competitive inhibitor of spermidine synthase, the enzyme responsible for the conversion of Put into Spd *(13)* (Fig. 2), and its use results in an increase in Put and Spm. MGBG blocks Spd and Spm synthesis by inhibiting *S*-adenosylmethionine decarboxylase (SAMD) activity (Fig. 2). Both CHA and MGBG also inhibit PMT and methyl-putrescine-oxidase (MPO), respectively. These are enzymes involved in the biosynthesis of tropane alkaloids (Fig. 1). However, when an inhibitor is in the presence of enzymes for which it has similar affinity, it is difficult to predict which inhibitory effect will predominate *(14)*.

The amino acid phenylalanine (Phe) also participates in the biosynthesis of these alkaloids, delivering the tropic acid moiety. However, Phe can be diverted into the phenylpropanoid pathway by virtue of the enzyme phenylalanine ammonia lyase (PAL) (Fig. 1). In the present work, *trans*-cinnamic acid was used to inhibit PAL, in an attempt to increase the Phe available for the tropane alkaloid pathway. Godoy-Hernandez and Loyola-Vargas *(15)*, working with *Catharanthus roseus* cell cultures, used *trans*-cinnamic acid to this end with promising results. Brincat et al. *(16)* used a similar approach in plant cell cultures of *Taxus canadiensis*, but in that case inhibition of taxol production ensued.

In this article, we report on the modifications observed in the production, release, and profile of scopolamine and hyoscyamine in hairy roots of

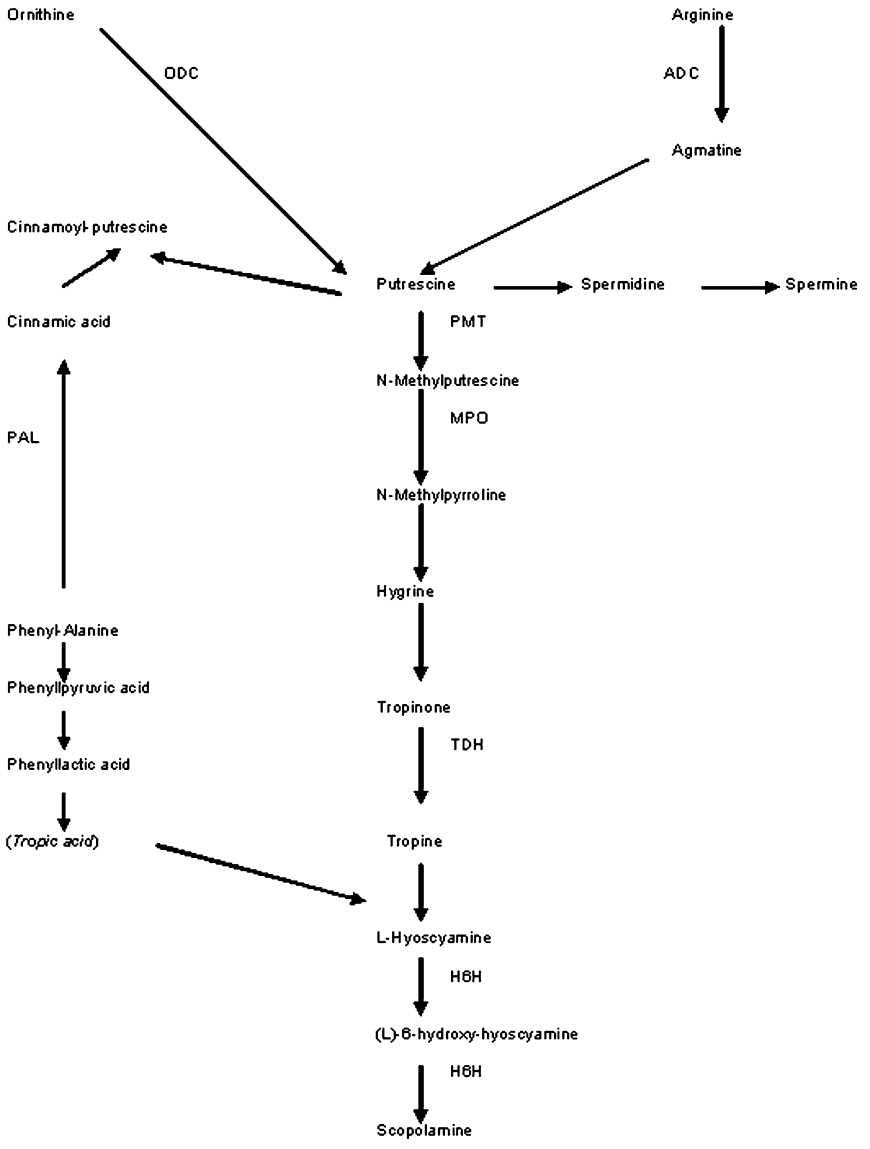

Fig. 1. Biosynthesis of hyoscyamine and scopolamine: ODC, ornithine-decarboxylase; ADC, arginine-decarboxylase; TDH, tropinone dehydrogenase; H6H, hyoscyamine 6-β-hydroxylase.

B. candida treated with the enzyme inhibitors CHA and MGBG. In addition, we discuss morphologic changes observed with CHA.

Materials and Methods

Chemicals

Scopolamine, l-hyoscyamine, cinnamic acid, CHA, MGBG, and all the medium components were purchased from Sigma (St. Louis, MO).

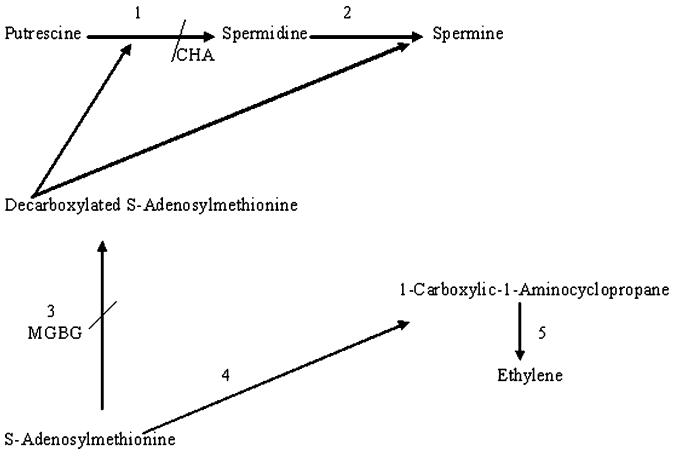

Fig. 2. Biosynthesis of polyamines and ethylene and inhibitors of polyamine synthesis: 1, spermidine synthase (putrescine aminopropyltransferase); 2, spermine synthase (spermidine aminopropyltransferase); 3, SAMD; 4, ACC synthase; 5, ethylene-forming enzyme.

Establishment of Hairy-Root Cultures

Hairy-root cultures were obtained by infecting explants of *B. candida* with *Agrobacterium rhizogenes* LBA 9402 following the procedure described in Pitta-Alvarez and Giulietti *(2)*. Establishment of the cultures and confirmation of transformation were done according to Pitta-Alvarez and Giulietti *(2)*. The roots were maintained in hormone-free Gamborg et al. *(17)* liquid medium with a half concentration of mineral salts and vitamins ($B5_{1/2}$) and supplemented with 15 g/L of sucrose. The roots were subcultured in the same medium every 20 d and 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 approx 1.8 W/m^2.

Time Course of Growth and Tropane Alkaloid Content

Inocula of fresh weight (0.5 g) of 20-d-old hairy roots were inoculated in 50 mL of $B5_{1/2}$ (with 15 g/L of sucrose) contained in 250-mL Erlenmeyer flasks. The cultures were incubated as just described, and samples were taken every 5 d for 30 d. Fresh weight and scopolamine and hyoscyamine content were determined for each sample.

Addition of CHA and MGBG at Different Stages of Growth Cycle

To study the effect of polyamine synthesis inhibitors, 50–100 mg of 15-d-old root tips was transferred to 25 mL of $B5_{1/2}$ medium supplemented with 15 g/L of sucrose contained in 125-mL Erlenmeyer flasks. Final concentrations of 2.5 m*M* CHA or 1.0 m*M* MGBG (both filter sterilized) were added to the cultures in three different stages of the growth cycle: (1) culture initiation (d 0), (2) beginning of exponential phase (d 10), and

(3) midexponential phase (d 20). All the flasks were harvested at d 25. Fresh weight and hyoscyamine and scopolamine in the roots and the medium were determined. All the experiments were done in triplicate.

Addition of CHA, MGBG and L-Cinnamic Acid During Exponential Phase

After 18 d of culture, CHA, MGBG, and *trans*-cinnamic acid were added according to the following scheme: (1) 2.5 m*M* CHA, (2) 1.0 m*M* MGBG, (3) 1.0 m*M* *trans*-cinnamic acid, (4) 2.5 m*M* CHA and 1.0 m*M* *trans*-cinnamic acid, and (5) 1.0 m*M* MGBG and 1.0 m*M* *trans*-cinnamic acid. The flasks were harvested after 48 h of culture. Fresh weight and hyoscyamine and scopolamine in the roots and the medium were determined. All the experiments were done in triplicate.

Effects of CHA on Root Morphology

To examine the effects of CHA on elongation and ramification, root tips (10 mm) were cultured on $B5_{1/2}$ medium supplemented with 15 g/L of sucrose and solidified with 0.5% agar. CHA was added at a final concentration of 2.5 m*M*. On d 5, 10, and 20, the growth of primary and lateral roots was measured and the number of lateral roots was counted.

Analytical Methods

Fresh weight was determined by separating the root tissue from the medium by vacuum filtration. Alkaloid extraction was carried out as described by Parr et al. *(18)*. Hyoscyamine and scopolamine were analyzed by high-performance liquid chromatography according to the method described by Mano et al. *(19)*.

Statistical Analysis

Significance of treatment effects was determined using analysis of variance. Variations among treatment means were analyzed using Tukey's *(20)* procedure ($p = 0.05$).

Results and Discussion

Characterization of Growth and Tropane Alkaloid Content

Figure 3 shows the time course of the growth of the hairy roots of *B. candida* employed. The lag phase was relatively short (5 d), and the specific growth rate in the exponential phase (μ_{max}) was 0.09 d^{-1}. Figure 4 shows the time course of scopolamine and hyoscyamine production. The accumulation of scopolamine and hyoscyamine increased during the exponential phase (15–20 d) and remained high during the stationary phase. The maximum productivity obtained for both scopolamine and hyoscyamine was approx 6 mg/L. Although this is lower than the values obtained by

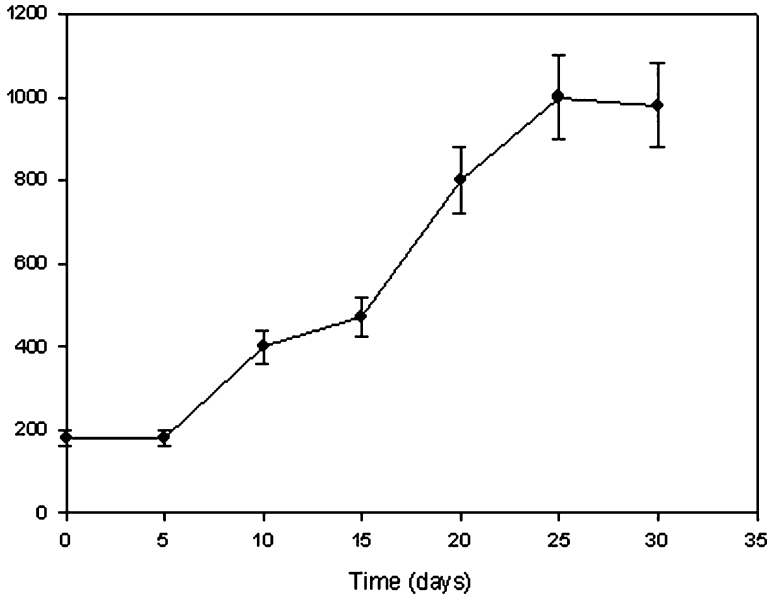

Fig. 3. Time course of growth of hairy roots of *B. candida*.

other investigators (e.g., Palazón et al. *[21]* obtained 20 and 7 mg/L, respectively), our objective was to determine whether the treatments tested influenced the production of these tropane alkaloids in our system of hairy roots.

Assays with CHA and MGBG Added at Different Stages of Growth Cycle

CHA

CHA had no effect on growth (data not shown). However, the accumulation of both hyoscyamine and scopolamine and their specific production decreased, in comparison to the controls (Fig. 5A). The negative effect on alkaloid production and not on growth suggests that CHA inhibited mainly PMT and not spermidine synthase. This is in agreement with results presented by Hibi et al. *(22)* in which CHA is referred to as a highly effective specific inhibitor of PMT.

On the other hand, the release of scopolamine into the medium was increased significantly with CHA treatment, especially when CHA was added in mid–exponential phase (Fig. 5B). The specific release of scopolamine was probably not a result of overproduction of the metabolite nor a result of an increase in putrescine or spermine, because polyamines in general increase membrane stability *(23,24)*. In any case, the reason that only scopolamine, and not hyoscyamine, is released remains to be elucidated.

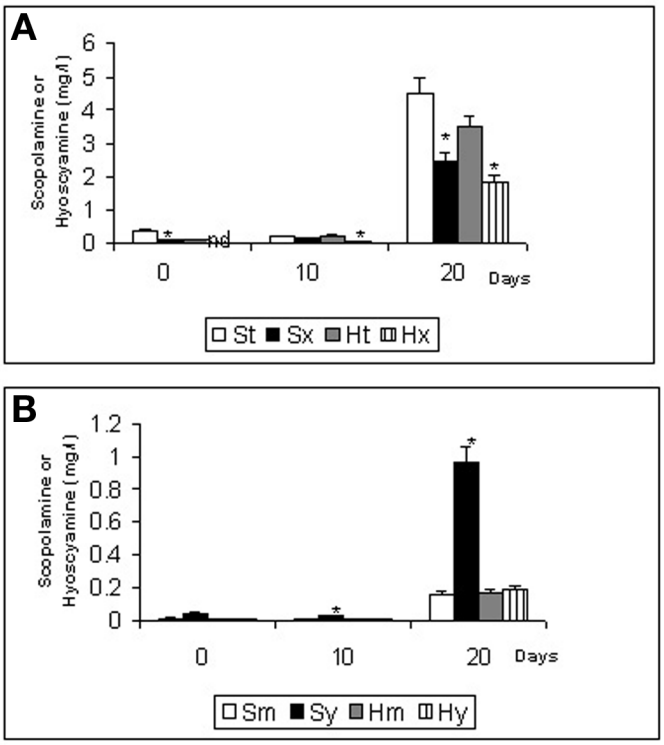

Fig. 4. Time course of accumulation (mg/L) of scopolamine (—●—) and hyoscyamine (—○—) in hairy roots of *B. candida*.

MGBG

MGBG inhibits SAMD, a key enzyme in the synthesis of spermidine (Fig. 2). MGBG had a strong negative effect on growth, particularly when it was added in the first stages of culture (data not shown). The roots treated with MGBG turned a dark brown, suggesting an increase in the formation of phenols, probably associated with senescence processes or as a response to stress. At the same time, also for the first stages of culture, the specific productivity was duplicated and the relationship between total scopolamine and total hyoscyamine (ratio St/Ht) increased significantly. Although MGBG also inhibits MPO, a diamine oxidase active in the tropane alkaloid pathway, the results suggest that its affinity for SAMD is superior. This is in agreement with studies carried out by Hashimoto et al. *(25)*, who demonstrated that MPO has a greater affinity for *N*-methylated amines (like *N*-methyl putrescine).

The most important effect observed with MGBG was an increase in the release of both scopolamine and hyoscyamine into the medium, particularly when the inhibitor was added in the first stages of culture (Fig. 6). This release could be owing to membrane damage or deficiencies in the formation of essential structures of the cell wall and/or membranes. In both these processes, polyamines, especially spermine *(9)*, play an

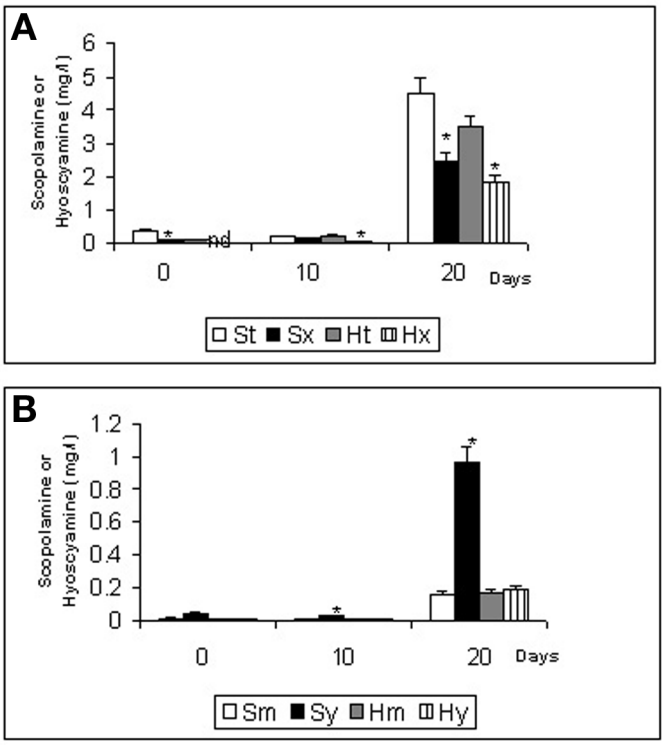

Fig. 5. Effect of 2.5 m*M* CHA added at different times of growth cycle on accumulation and release into medium of scopolamine and hyoscyamine in hairy roots of *B. candida*. The days refer to the time of the growth cycle when the inhibitor was added. **(A)** Total scopolamine or hyoscyamine (roots and medium); **(B)** scopolamine or hyoscyamine in medium. St, scopolamine control; Ht, hyoscyamine control; Sx, scopolamine in presence of inhibitor; Hx, hyoscyamine in presence of inhibitor; Hm, hyoscyamine control; Sm, scopolamine control; Sy, scopolamine in presence of inhibitor; Hy, hyoscyamine in presence of inhibitor; nd, not detected. Each value represents the mean of three independent determinations. Data marked with an asterisk are significantly different with respect to the corresponding control according to Tukey's test ($p = 0.05$).

essential role. In addition, treatment with MGBG can act positively on the synthesis of ethylene (Fig. 2). This could explain the decrease in biomass and the appearance of early indicators of senescence, such as the high levels of phenols. In turn, the negative effects of ethylene on membranes *(26)* could possibly not be counteracted by polyamines, because their synthesis is inhibited. Finally, MGBG also inhibits diamine oxidases. Although many of the functions for diamine oxidases are little known, it has been suggested that they are related to the integrity and rigidity of the cell wall *(27)*. MGBG could be inhibiting these enzymes and contributing to the permeabilization of the cell.

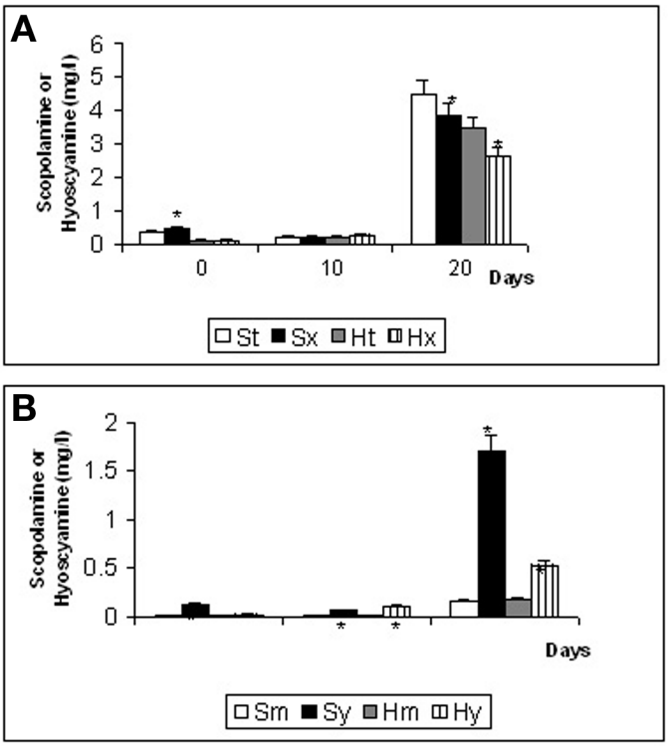

Fig. 6. Effect of 1.0 m*M* MGBG added at different times of growth cycle on accumulation and release into medium of scopolamine and hyoscyamine in transformed roots of *B. candida*. The days refer to the time of the growth cycle when the inhibitor was added. **(A)** Total scopolamine or hyoscyamine (roots and medium); **(B)** scopolamine or hyoscyamine in medium. St, scopolamine control; Ht, hyoscyamine control; Sx, scopolamine in presence of inhibitor; Hx, hyoscyamine in presence of inhibitor; Hm, hyoscyamine control; Sm, scopolamine control; Sy, scopolamine in presence of inhibitor; Hy, hyoscyamine in presence of inhibitor; nd, not detected. Each value represents the mean of three independent determinations. Data marked with an asterisk are significantly different with respect to the corresponding control according to Tukey's test ($p = 0.05$).

Assays with CHA, MGBG, and trans-Cinnamic Acid Added During Exponential Phase

The following treatments were done during the exponential phase owing to the high tropane alkaloid–producing properties of this period. The time of exposure was low, in order to diminish the detrimental effects it could have on the plant material.

CHA

Treatment with CHA for 48 h during the exponential phase had opposite effects to those observed in the experiments just described. The accumulation and release of both alkaloids, particularly scopolamine, increased

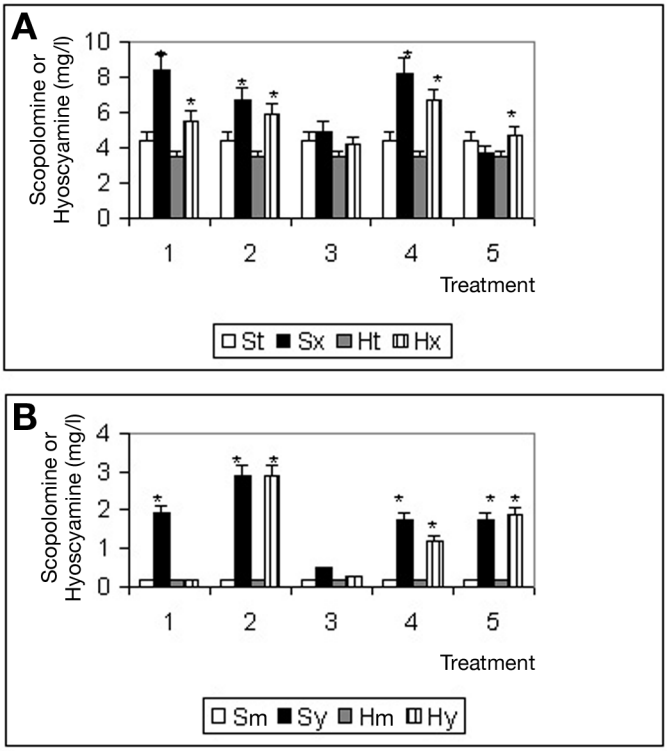

Fig. 7. Effect of , MGBG, and cinnamic acid on accumulation and release into medium of scopolamine and hyoscyamine in transformed roots of *B. candida*. **(A)** Total scopolamine or hyoscyamine (roots and medium); **(B)** scopolamine or hyoscyamine in medium. The three inhibitors were added to 18-d cultures (exponential phase) and the exposure time was 24 h. Treatments were as follows: (1) 2.5 m*M* CHA; (2) 1.0 μ*M* MGBG; (3) 1.0 μ*M* cinnamic acid; (4) 2.5 μ*M* CHA + 1.0 μ*M* cinnamic acid; (5) 2.5 μ*M* MGBG + 1.0 μ*M* cinnamic acid. St, scopolamine control; Ht, hyoscyamine control; Sx, scopolamine in presence of inhibitor(s); Hx, hyoscyamine in presence of inhibitor(s); Hm, hyoscyamine control; Sm, scopolamine control; Sy, scopolamine in presence of inhibitor(s); Hy, hyoscyamine in presence of inhibitor(s). Each value represents the mean of three independent determinations. Data marked with an asterisk are significantly different with respect to the corresponding control according to Tukey's test ($p = 0.05$).

significantly (Fig. 7). The specific productivity of both increased dramatically, and the ratio St/Ht clearly benefited scopolamine. The reasons for these important discrepancies have not been elucidated but are probably related to the lower time of exposure to the inhibitor.

MGBG

MGBG did not affect growth (data not shown), but all the other parameters were in accordance with the ones observed in the previous assays. Accumulation of both metabolites in the roots slightly decreased (Fig. 7). The release into the medium and the specific productivity of both metabolites increased significantly (Fig. 7).

Cinnamic Acid

Cinnamic acid negatively affected growth (data not shown) but in turn increased the release of both alkaloids into the medium, especially scopolamine (Fig. 7B). The release could be owing to the fact that PAL plays an important role in the integrity of plant cell walls, through the synthesis of phenols and their interaction with peroxidases *(28)*.

CHA/Cinnamic Acid

The combination CHA/cinnamic acid had a positive effect on the release into the medium, especially hyoscyamine, and on the specific productivity of both alkaloids (Fig. 7B). The accumulation in roots was also augmented, favoring hyoscyamine (Fig. 7A).

MGBG/Cinnamic Acid

The combination MGBG/cinnamic acid did not affect the accumulation of both metabolites in the roots (Fig. 7A). However, the release of both into the medium was increased dramatically, although to a lesser extent than when MGBG was added alone (Fig. 7B). There was also an increase in the specific productivity of both alkaloids. Furthermore, a decrease in the ratio St/Ht was also observed.

In the treatment with cinnamic acid, the increase in endogenous phenylalanine did not result in an increase in the production of tropane alkaloids. It has been suggested that cinnamoyl-putrescine could be a precursor in the synthesis of hyoscyamine in root cultures of *Hyoscyamus muticus (29)*. In this case, the deficit of cinnamic acid, as a result of PAL inhibition, could have been decisive.

Effects of CHA on Root Morphology

The results in Table 1 demonstrate that CHA had important effects on the morphology of transformed roots of *B. candida*. The growth of primary roots was negatively influenced by CHA. Lateral roots, however, were positively affected. Although CHA had an inhibitory effect on ramification, it increased the elongation of lateral roots. By contrast, Biondi et al. *(30)*, working with hairy roots of *H. muticus*, found that CHA repressed overall root growth.

The literature indicates that polyamines play an important role in primary, lateral, and adventitious root development *(31–35)*. However, the discrepancies among results highlight important interspecies differences and dependence on culture conditions *(36)*. There is, though, clear evidence that in the roots of different species Put increases as elongation progresses and is higher in differentiating zones, whereas Spm and Spd are most abundant near apices. Thus, the increase in Put and the depletion of Spd caused by CHA could explain the prevalence of elongation processes as opposed to differentiation ones.

Table 1
Effect of CHA and MGBG on Elongation Rate and Number and Length of New Ramifications in Transformed Roots of *B. candida*[a]

	Day 5				Day 10				Day 20			
	A	B	C	D	A	B	C	D	A	B	C	D
Control	0.2[a]	0.6[a]	3.1[a]	—	0.9[a]	1.0[a]	5.2[a]	25[a]	1.6[a]	0.5[a]	21[a]	30[a]
CHA (2.5 m*M*)	0.2[a]	1.0[b]	3.0[a]	—	0.1[b]	1.5[b]	4.3[a]	60[b]	0.1[b]	2.0[b]	8[b]	70[b]
MGBG (1.0 m*M*)	0.2[a]	0.7[a]	1.5[b]	—	0[c]	0[c]	0[c]	—	0[c]	0[c]	0[c]	—

[a]Each value represents the mean of 10 independent determinations that typically varied by 10%. Data with the same letter in each column were not significantly different according to Tukey's test ($p = 0.05$). A, rate of elongation (mm/d) of primary roots; B, rate of elongation (mm/d) of lateral roots; C, number of new ramifications (each value refers to the number of preexisting ramifications at the beginning of the culture period, which was assigned an arbitrary value of 1); D, length (mm) of the longest ramifications.

Conclusion

From the results described, it can be concluded that treatment with polyamine biosynthesis inhibitors, particularly CHA, can be favorable to production without being deleterious to growth. Palazón et al. *(21)* reported final scopolamine contents of up to 75 mg/L, whereas we obtained a maximum of 12 mg/L. However, it must be taken into account that their control values were higher than ours (24 mg/L) and that their approach to increase scopolamine production was the overexpression of the hyoscyamine-6-β-hydroxylase gene. As far as the ratio scopolamine/hyoscyamine is concerned, we found that under optimized conditions it was clearly favorable to scopolamine, which is the most economically valuable of the two alkaloids *(37)*. It is important to highlight the fact that this variable is susceptible to a myriad of factors, among which we can cite nitrate and ammonium concentrations *(38)* and elicitation *(39)*.

Acknowledgments

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