Occurrence of cadaverine in hairy roots of *Brugmansia candida*

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Abstract

The polyamine, cadaverine, was detected in transformed root cultures of *Brugmansia candida* (syn. *Datura candida*), a Solanaceae which produces the tropane alkaloids scopolamine and hyoscyamine. To the best of our knowledge, this is the first time that the existence of this uncommon polyamine has been detected in a *Datura* species. Cadaverine, however, could not be found in the whole plant. The occurrence of cadaverine in hairy roots could be a consequence of either the transformation or a response to stress. Also, cadaverine could be participating in other secondary pathways rather than to the tropane alkaloids. The common polyamines, putrescine, spermidine and spermine were also observed.

Keywords: *Brugmansia candida* (syn. *Datura candida*); Solanaceae; Cadaverine; *Agrobacterium rhizogenes*; Hairy roots; Polyamines

1. Introduction

Brugmansia candida (syn. *Datura candida*), a member of the Solanaceae, is a South American plant that produces the tropane alkaloids scopolamine and hyoscyamine (Roses et al., 1988). These alkaloids are extensively employed in medicine as anticholinergic agents. In our laboratory, we have worked with transformed (hairy) roots of *B. candida* in order to obtain these alkaloids (Pitta-Alvarez and Giulietti, 1995, 1997). The biosynthesis of these secondary metabolites uses the polyamine putrescine (Put) as a precursor (Scheme 1), a compound which can also diverge into the metabolic route leading to two other polyamines, spermidine (Spd) and spermine (Spm), both linked mainly to primary metabolism in this plant.

The common aliphatic polyamines Put, Spd and Spm, are ubiquitous in all types of organisms, and are considered to participate in a variety of events related to plant growth and development (Bagni and Torrigiani, 1992; Galston and Kaur Sawhney, 1995). However, besides these common polyamines, there are also some uncommon ones, such as 1,3-diaminopropane, cadaverine (Cad), norspermidine and homospermidine, which have been associated with the capacity of some biological systems to grow or function under extreme environments (Slocum and Flores, 1991). Cad (1,5-diaminopentane), is the penta homologue of Put, and it is present in many biological tissues. However, the existence of this diamine in plants is sporadic and less common than its homologue Put. Cad has been found in several genera of the Leguminosae, such as *Pisum*, *Glycine*, *Lathyrus*, *Trifolium*, *Lupinus* and *Vicia*; in *Hordeum* and *Oryza* (Gramineae), in *Zantedeschia* (Araceae), and in *Sedum* (Crassulaceae). In the Solanaceae, however, it has only been detected in *Nicotiana* and *Lycopersicon* (Slocum and Flores, 1991; Wink 1997).

The objective of our work was to evaluate the polyamine (Put, Spd and Spm) levels in hairy roots of *B. candida*. Unexpectedly, Cad was also found.

Abbreviations: Cad, Cadaverine; Put, putrescine; Spd, spermidine; Spm, spermine

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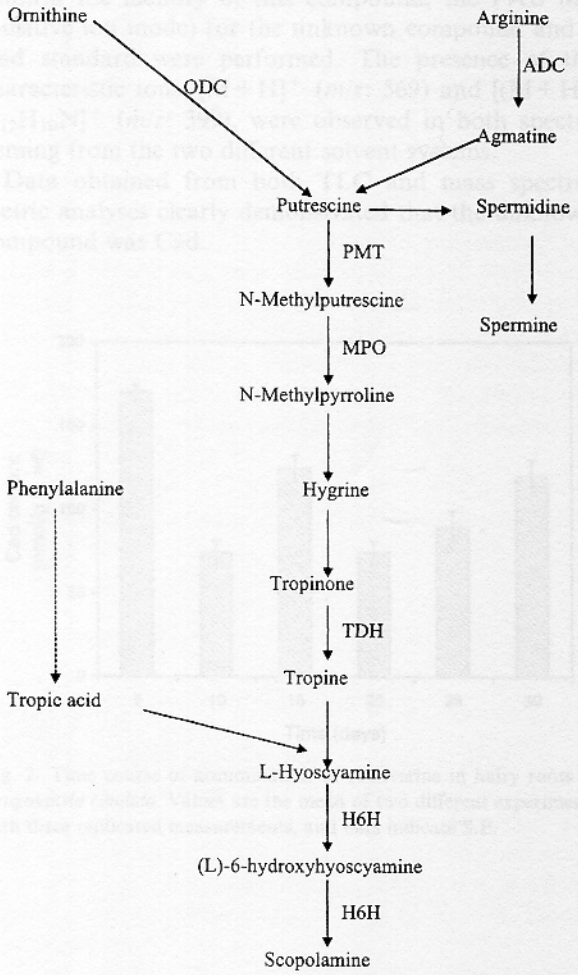
2. Results and discussion

2.1. Time-course of growth and polyamine biosynthesis

Table 1 and Fig. 1 show the time course of growth of hairy root cultures of *B. candida* and the corresponding Put, Spd and Spm contents, respectively. The values obtained were similar to those observed by Robins et al. (1991) in hairy roots of *Datura stramonium*. The levels of the three polyamines decrease when the cultures enter into an exponential phase. This depletion could suggest that they are being used actively for cell division and growth.



Scheme 1. Biosynthesis of hyoscyamine and scopolamine. ODC: ornithine-decarboxylase; ADC: arginine-decarboxylase; PMT: putrescine-methyltransferase; MPO: methylputrescine-oxidase; TDH: tropinone dehydrogenase; H6H: hyoscyamine 6-β-hydroxylase.

Table 1
Time course of growth of *Brugmarsia candida* hairy roots[a]

Time (days)	Fresh wt (g)
5	0.22±0.02
10	0.51±0.05
15	0.47±0.05
20	1.02±0.01
25	1.7±0.12
30	1.61±0.11

[a] Data are mean values of two independent experiments±S.E. Each point represents six replicates.

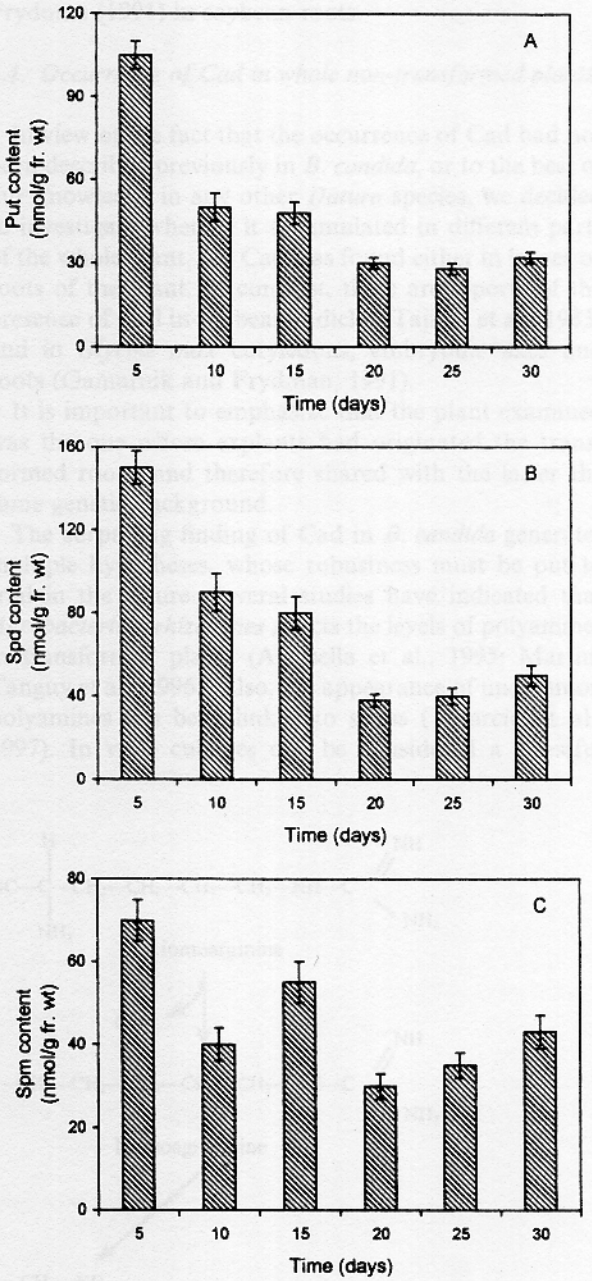


Fig. 1. Time course of accumulation of polyamines: (A) putrescine, (B) spermidine and (C) spermine in hairy roots of *Brugmarsia candida*. Values are the mean of two different experiments with three replicated measurements, and bars indicate S.E.

2.2. Cadaverine detection

When the TLCs of the perchloric extracts obtained from the cultures were examined, an additional band, which had the same R_f (in chloroform:triethylamine) as Cad, was detected. Moreover, this compound showed the same R_f as Cad in *n*-hexane:ethyl acetate. To confirm the identity of this compound, the FAB MS (positive ion mode) for the unknown compound and a Cad standard were performed. The presence of the characteristic ions, $[M+H]^+$ (*m/z*: 569) and $[(M+H)-C_{12}H_{10}N]^+$ (*m/z*: 399), were observed in both spectra coming from the two different solvent systems.

Data obtained from both TLC and mass spectrometric analyses clearly demonstrated that the unknown compound was Cad.

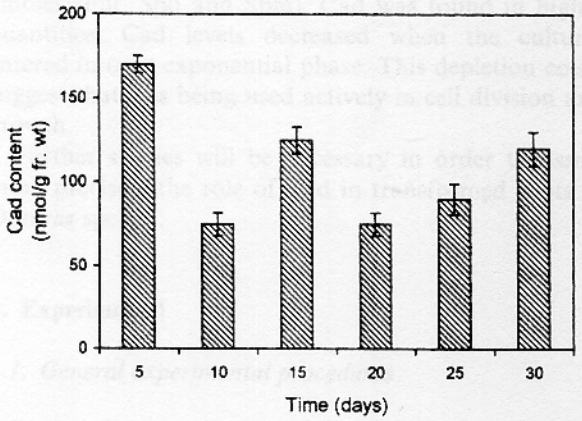


Fig. 2. Time course of accumulation of cadaverine in hairy roots of *Brugmansia candida*. Values are the mean of two different experiments with three replicated measurements, and bars indicate S.E.

2.3. Time-course of growth and cadaverine content

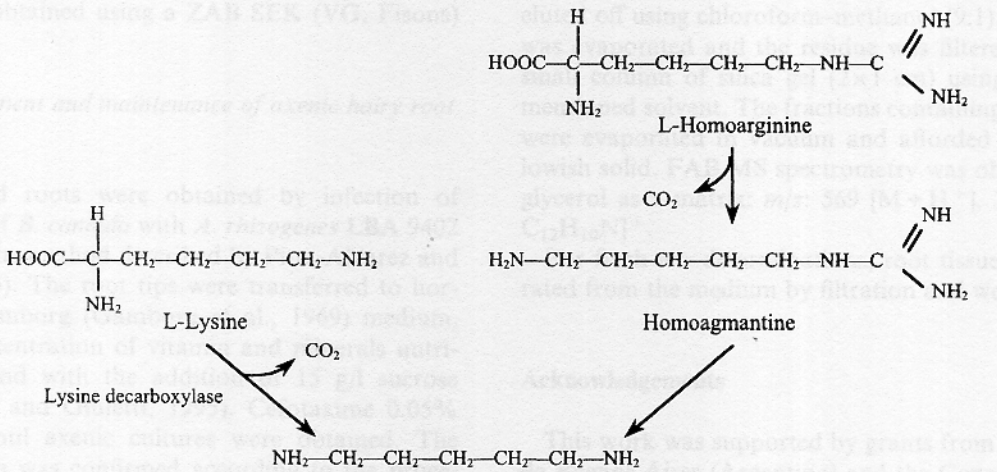
Table 1 and Fig. 2 show the time course of growth of hairy root cultures of *B. candida* and the corresponding Cad content, respectively. In comparison to the other three polyamines (Fig. 1), Cad was found in slightly higher quantities. However, the same phenomena of reduction of polyamine content concomitant with the exponential growth period, was observed. These results are in agreement with those found by Gamarnik and Frydman (1991) in soybean roots.

2.4. Occurrence of Cad in whole non-transformed plants

In view of the fact that the occurrence of Cad had not been described previously in *B. candida*, or to the best of our knowledge in any other *Datura* species, we decided to investigate whether it accumulated in different parts of the whole plant. No Cad was found either in leaves or roots of the plant. In contrast, there are reports of the presence of Cad in soybean radicles (Tajima et al., 1983) and in *Glycine max* cotyledons, embryonic axes and roots (Gamarnik and Frydman, 1991).

It is important to emphasize that the plant examined was the one whose explants had originated the transformed roots, and therefore shared with the latter the same genetic background.

The surprising finding of Cad in *B. candida* generates multiple hypotheses, whose robustness must be put to trial in the future. Several studies have indicated that *Agrobacterium rhizogenes* affects the levels of polyamines in transformed plants (Altabella et al., 1995; Martin-Tanguy et al., 1996). Also, the appearance of uncommon polyamines has been linked to stress (Tiburcio et al., 1997). In vitro cultures can be considered a stressful



Scheme 2. Biosynthesis of cadaverine.

situation for plants, and the expression of the gene coding for the enzyme that synthesizes Cad could be triggered by it (Flores, 1991) either for transforming lysine into Cad in a reaction catalyzed by lysine decarboxylase or via an alternative route that involves L-homoarginine and homoagmatine (Wink, 1997) (Scheme 2).

Finally, the main question that remains unanswered is what is the role of Cad in hairy roots. As was mentioned previously, it could be part of the roots response to stress. Also, their participation in secondary routes, not necessarily to tropane alkaloids, might be possible.

3. Conclusions

Cadaverine (Cad), an uncommon polyamine, was detected for the first time in hairy roots of *Brugmansia candida*, and in comparison to the other three polyamines (Put, Spd and Spm), Cad was found in higher quantities. Cad levels decreased when the cultures entered into an exponential phase. This depletion could suggest that it is being used actively in cell division and growth.

Further studies will be necessary in order to assess more precisely the role of Cad in transformed roots of *Daturas* species.

4. Experimental

4.1. General experimental procedures

TLC was performed on high resolution silica gel plated (Merck, silica gel 60 F-254 plates). The silica gel used in column chromatography was Kieselgel DG (Riedel de Haen). All the chemical used were of analytical grade. Solvents were distilled before use. FAB MS spectra were obtained using a ZAB SEK (VG, Fisons) spectrometer.

4.2. Establishment and maintenance of axenic hairy root cultures

Transformed roots were obtained by infection of sterile leaves of *B. candida* with *A. rhizogenes* LBA 9402 according to the method described by Pitta-Alvarez and Giulietti (1995). The root tips were transferred to hormone-free Gamborg (Gamborg et al., 1969) medium, with half concentration of vitamin and minerals nutrients ($B5_{1/2}$) and with the addition of 15 g/l sucrose (Pitta-Alvarez and Giuletti, 1995). Cefotaxime 0.05% was added until axenic cultures were obtained. The transformation was confirmed according to the procedure described by Hamill et al. (1991). The roots that were used in the experiments were maintained in an hormone-free $B5_{1/2}$ medium, supplemented with 15 g/l sucrose and subcultured every 15–20 days. 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 W m^{-2}.

4.3. Time course of growth and polyamine content

Inocula of fresh weight (0.5 g) of 20-day-old hairy roots were inoculated in 50 ml of $B5_{1/2}$ (with 15 g/l sucrose) contained in 250 ml Erlenmeyers. The cultures were incubated as described above and samples were taken every 5 days during 30 days and fresh wt and free polyamines were determined in each one of them.

4.4. Analytical methods

Plant material (400 mg fresh wt of hairy roots, leaves and normal roots) was homogenized with 5% perchloric acid, kept 30 min on ice and centrifuged at 5000 rpm for 10 min. The supernatants (15 ml) were derivatized using the dansylation method described by Smith and Meeuse (1966) and 1,6 hexanediamine was used as internal standard. Standards of Put, Spd, Spm were dansylated simultaneously. The dansylated derivatives were extracted with 1 ml ethyl acetate. Polyamines (200 µl) were separated and identified by TLC with both chloroform–triethylamine (9:1) and *n*-hexane–ethyl acetate (2:1) solvent systems. Dansylated polyamines were identified by comparing the R_f values of dansylated standards. Silica plates were observed under UV light and the bands corresponding to polyamines in the samples and standards were scraped off the plates and eluted with EtOAc (1 ml). Their fluorescence was measured in a spectrofluorometer using an excitation wavelength of 365 nm and an emission wavelength of 510 nm.

For confirmation of the identity of Cad, the band with the same R_f as Cad was scraped from the plate and eluted off using chloroform–methanol (9:1). The solvent was evaporated and the residue was filtered through a small column of silica gel (2×1 cm) using the above-mentioned solvent. The fractions containing the product were evaporated in vacuum and afforded a white yellowish solid. FAB MS spectrometry was obtained using glycerol as a matrix: *m/z*: 569 $[M+H^+]$, 399$[(M+H)-C_{12}H_{10}N]^+$.

For fresh wt determinations, root tissues were separated from the medium by filtration and weighed.

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