



Article scientifique

Article

1998

Published version

Public access

This is the published version of the publication, made available in accordance with the publisher's policy.

Hairy roots of *Datura candida* × *D. aurea*: effect of culture medium composition on growth and alkaloid biosynthesis

Nussbaumer, Philippe; Kapetanidis, Ilias; Christen, Philippe

How to cite

NUSSBAUMER, Philippe, KAPETANIDIS, Ilias, CHRISTEN, Philippe. Hairy roots of *Datura candida* × *D. aurea*: effect of culture medium composition on growth and alkaloid biosynthesis. In: Plant cell reports, 1998, vol. 17, n° 5, p. 405–409. doi: 10.1007/s002990050415

This publication URL: <https://archive-ouverte.unige.ch/unige:172752>

Publication DOI: [10.1007/s002990050415](https://doi.org/10.1007/s002990050415)

© The author(s). This work is licensed under a Backfiles purchase (National Licenses Project) <https://www.unige.ch/biblio/aou/fr/guide/info/references/licences/>

Last update in Archive ouverte UNIGE on 08.11.2023 11:55

P. Nussbaumer · I. Kapétanidis · P. Christen

Hairy roots of *Datura candida* × *D. aurea*: effect of culture medium composition on growth and alkaloid biosynthesis

Received: 12 March 1997 / Revision received: 22 April 1997 / Accepted: 5 May 1997

Abstract A transformed root clone of *Datura candida* × *D. aurea* was established following infection with *Agrobacterium rhizogenes* strain A4. This clone was examined for its growth and hyoscyamine and scopolamine content under various culture conditions. Among the three basal culture media tested, half-strength Gamborg's B5 medium supplemented with 5% sucrose was the best for root growth (288 mg dry weight/flask) and full-strength B5 medium for hyoscyamine and scopolamine content (0.36 and 0.17% dry weight, respectively). Experiments with exogenous nitrate added to the medium revealed that the biomass increased (353 mg dry weight/flask) and the hyoscyamine content improved remarkably (0.54% dry weight), but that the scopolamine content was significantly reduced. The addition of various precursors at two different concentrations did not significantly modify root growth. Feeding (R,S)-phenyllactic acid stimulated the biosynthesis of both alkaloids, whereas the addition of ornithine specifically reduced the scopolamine content.

Key words *Datura candida* × *D. aurea* · Transformed roots · Hyoscyamine · Scopolamine · Medium optimization

Abbreviations MS Murashige and Skoog medium · N/A nitrate/ammonium · WP McCown's woody plant medium

Introduction

Several species belonging to the genus *Datura* are cultivated for their tropane alkaloid content. Recently, there has

been considerable interest in the production of this class of compounds by genetically transformed root cultures. In addition, hybridization offers a means to develop new chemical varieties of plants, possibly superior to either of the parents. The genus *Datura* has received some attention with respect to alkaloid production with the research work of Evans (1979). The hybrid possibility has been investigated by crossing *Datura candida*, a white-flowered tree datura, with *D. aurea*, a closely related yellow-flowered form (El-Dabbas and Evans 1982, El-Imam and Evans 1990). The alkaloid content in the aerial parts of the F₁ generation of *D. candida* × *D. aurea* reached 0.68–0.72% (dry weight) scopolamine and 0.17–0.19% (dry weight) hyoscyamine. The combination of traditional breeding methods with modern plant biotechnology may thus find application in the improvement of medicinal plants.

Transformed root cultures of *D. candida* × *D. aurea* have already been reported by Robins et al. (1990). With GC-MS they identified 36 tropane alkaloids and derivatives, among which hyoscyamine and scopolamine were the main compounds: 0.046% (fresh weight) and 0.030% (fresh weight), respectively. The activities of six enzymes were also investigated to determine the probable factors limiting metabolic flux in the biosynthetic pathway. However, the culture medium had not been optimized.

As part of our investigations into the production of hyoscyamine and scopolamine by tissue cultures (Christen et al. 1989, 1992; Dupraz et al. 1994), we report here on the establishment of hairy roots of *D. candida* × *D. aurea* by inoculation with *Agrobacterium rhizogenes* strain A4 and the examination of culture conditions. Factors influencing root growth and hyoscyamine and scopolamine production are evaluated and discussed.

Materials and methods

Bacteria and plant material

A. rhizogenes strain A4 (pRiA4) was kindly provided by Dr. Kamada of Tsukuba University, Ibaraki, Japan. The bacteria were grown

Communicated by P. Matile

P. Nussbaumer · I. Kapétanidis · P. Christen (✉)
Laboratory of Pharmaceutical Analytical Chemistry,
University of Geneva, 20, bd D'Yvoy,
CH-1211 Geneva 4, Switzerland
Fax no: +41-22-781-51-93
E-mail: Philippe.Christen@pharm.unige.ch

on solid yeast mannitol broth (Ooms et al. 1985) medium and subcultured at 1-month intervals. Seeds (F_1 generation) of *D. candida* $\times$ *D. aurea* were a generous gift of Dr. Evans from the University of Nottingham, UK.

Establishment of hairy root cultures

Seeds were surface sterilized for 12 min in a 2% (wt/vol) sodium hypochlorite solution supplemented with two drops of Triton X-100 (Fluka, Switzerland), thoroughly rinsed with sterile distilled water and sown on wet filter paper in Petri dishes in darkness at 26°C. One week after germination, the plantlets were transferred to solid hormone-free Murashige and Skoog (1962) (MS) medium (Sigma, USA) containing 2% sucrose and adjusted to pH 5.8 before autoclaving, and cultivated at 26°C with a daily 16 h photoperiod (light source: Sylvania Gro-Lux, 700 lux).

The stems of 17-day-old sterile plantlets were infected with *A. rhizogenes* and 11 days after inoculation, the first hairy roots appeared at the infection sites. Root segments 10–15 mm long were cut off and transferred into hormone-free half-strength Gamborg (1970) B5 liquid medium (Sigma, USA) containing 3% sucrose (1/2 B5-3) and 0.25 g/l cefotaxime and 1 g/l ampicillin (Sigma, USA). After elimination of the bacteria, the hairy roots were inoculated in 1/2 B5-3 (50 ml in 250-ml conical flasks), cultured in darkness at 26°C on gyratory shakers at 80 rpm and subcultured at 4-week intervals.

Opine detection

Agropine, agropinic acid, mannopine, and mannopinic acid were analyzed by paper electrophoresis essentially according to Petit et al. (1983).

Analysis of alkaloids

The hairy roots were harvested and the fresh and dry weights after lyophilization were determined. Typically, about 40 mg (dry weight) were extracted with a 50-ml mixture of CH_2Cl_2 :MeOH:25% NH_4OH (15:5:1) for 20 min. After centrifugation, the residue was washed successively with 5 ml MeOH and 5 ml CH_2Cl_2 and the combined filtrates were evaporated, redissolved in 5 ml CH_2Cl_2 , mixed with 2 ml 5% NH_4OH and concentrated to a volume of 2 ml. The solution was applied onto an Extrelut-3 column (Merck, Germany) and eluted with 25 ml CH_2Cl_2 . The organic eluate was evaporated to dryness and the residue was dissolved in 1 ml MeOH. Half of the solution was evaporated under nitrogen and the residue was dissolved in 1.0 ml CH_3CN containing 0.005% (m/v) benzylamine (Fluka, Switzerland) as internal standard. The content of hyoscyamine and scopolamine was analyzed by HPLC on a Nucleosil 100–5 CN stainless steel column [25 cm long $\times$ 4 mm internal diameter (i.d.)] fitted with a Nucleosil 100 CN (11 mm $\times$ 4 mm i.d.) precolumn (Macherey-Nagel, Germany). After injection of 20 μl of sample solution, the column was eluted isocratically at 30°C with a mixture of CH_3CN :acetate buffer (95:5) at a flow rate of 1 ml/min. The buffer containing 0.06 M ammonium acetate and 0.03 M acetic acid was adjusted to pH 4.0 with hydrochloric acid. Hyoscyamine and scopolamine were detected at 210 nm. For quantitative analysis, the system was calibrated with reference alkaloids obtained commercially (Sigma). Standard curves were fitted with linear regression and their linearity was tested (Geigy Scientific Tables 1982). Controls containing known amounts of alkaloids were also set up to determine the efficiency of the extraction process. The mean extraction rates were 95.5% for hyoscyamine and 98.5% for scopolamine. Specificity of the method was verified by thermospray liquid chromatography-mass spectrometry (Nussbaumer 1996). Liquid medium (50 ml) was basified to pH 12 with 25% NH_4OH and extracted with 8 ml CH_2Cl_2 (three times) as described above and analyzed using the same HPLC conditions.

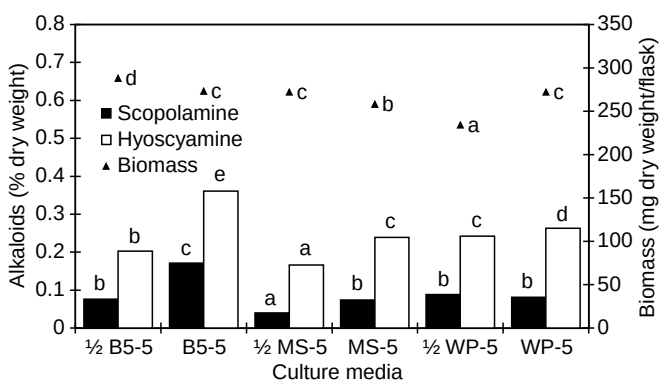


Fig. 1 Growth and alkaloid content in *Datura candida* $\times$ *D. aurea* hairy roots cultured in different liquid media for 4 weeks. Values with different lower-case letters are significantly different at $P < 0.05$

Statistical analysis and presentation of the results

Statistical analysis ($P=0.05$) was carried out according to Bartlett's test (homogeneity of the variance) and the results were classified according to Duncan's multiple-range test (Geigy Scientific Tables 1982). Means followed by the same letter on the histograms are not significantly different from each other. In all cases, values represent the means of five replicate flasks for each treatment, each flask assayed in triplicate.

Results and discussion

Effects of basal medium

The selected hairy root clone of *D. candida* $\times$ *D. aurea* was cultured in different growth media supplemented with 5% sucrose for 4 weeks, and examined for growth and for hyoscyamine and scopolamine content. The media tested were B5, MS and McCown's woody plant medium (Lloyd and McCown 1980) (WP) at half and full strengths. As shown in Fig. 1, root growth was not strongly affected by the different culture media. Maximum growth was obtained when the roots were cultured in half-strength B5 medium (288 mg dry weight) whereas the biomass was significantly lower in half-strength WP-5 (234 mg dry weight). In the four other media, intermediate growth was observed. In contrast, the culture media had a significant impact on the alkaloid content. In all cases, hyoscyamine was the main alkaloid. The highest concentrations of hyoscyamine and scopolamine were observed in B5-5 medium and reached 0.36 and 0.17% dry weight, respectively. Furthermore, this medium allowed a reasonable growth of the transformed roots (273 mg dry weight). Consequently, the alkaloid yield reached in this medium was 0.98 and 0.47 mg/flask for hyoscyamine and scopolamine, respectively. This medium also yielded the highest scopolamine levels, and the hyoscyamine/scopolamine ratio reached 2.22. In the other media, the hyoscyamine level fluctuated between 0.20% (dry weight) in 1/2 B5-5 and 0.26% (dry weight) in WP-5. The scopolamine content was more stable and fluctuated between 0.07 and 0.09% (dry weight), ex-

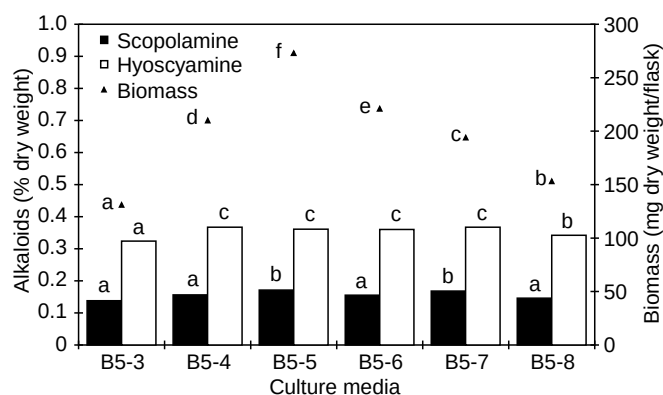


Fig. 2 Growth and alkaloid content in *D. candida* × *D. aurea* hairy roots cultured in B5 liquid medium with different sucrose concentrations for 4 weeks. Values for different lower-case letters are significantly different at $P < 0.05$

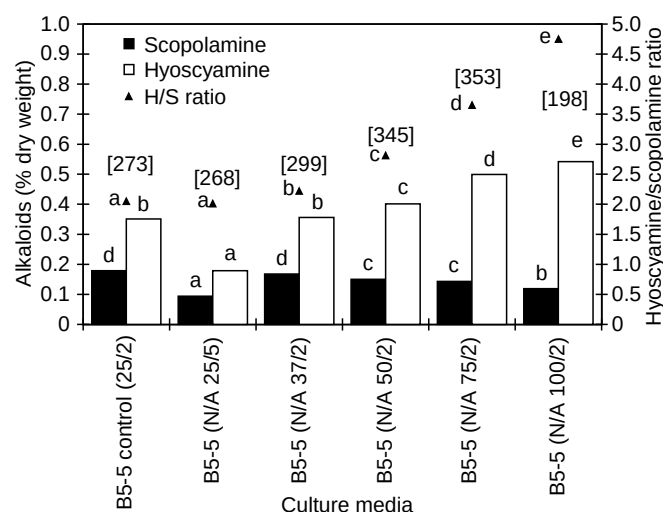


Fig. 3 Growth, alkaloid content and hyoscyamine/scopolamine (H/S) ratio in *D. candida* × *D. aurea* hairy roots cultured in B5 medium containing 5% sucrose and supplemented with various concentrations of NH_4^+ and NO_3^- for 4 weeks. Values in square brackets show the dry weight per flask (mg). Results with different lower-case letters are significantly different at $P < 0.05$

cept in 1/2 MS-5, in which it dropped to 0.04%. In these media, the hyoscyamine/scopolamine ratio varied between 2.81 (1/2 B5-5) and 4.40 (1/2 MS-5) and thus were not suitable conditions for high scopolamine content.

The culture media were also examined for alkaloid release. In all cases, no alkaloid was detected in the culture media. This result is in good agreement with those previously reported with hairy root cultures of other *Datura* species (Robins et al. 1990, Dupraz et al. 1994)

Effects of sucrose concentration

The hairy roots cultured in Gamborg B5 medium with sucrose concentrations between 3 and 8% were examined.

Results for biomass and alkaloid content after 28 days are shown in Fig. 2. Root growth was fastest in medium supplemented with 5% sucrose (273 mg dry weight). This is about two times higher than in medium containing 3% sucrose, for which the root growth was slowest (131 mg dry weight). On the other hand, the alkaloid content was not strongly affected by the different sucrose concentrations. There was no significant difference in the hyoscyamine content between media supplemented with 4–7% sucrose (0.37% dry weight), whereas the level was 0.32% (dry weight) in 3% sucrose and 0.34% (dry weight) in 8% sucrose. The scopolamine content was found to be very stable independently of the sucrose concentration and fluctuated between 0.14% (dry weight) in B5-3 and 0.17% (dry weight) in B5-5. Therefore, there is no direct influence of sucrose on the biosynthetic capacity or the hyoscyamine/scopolamine ratio of the root cultures. On the basis of these results, B5 medium supplemented with 5% sucrose was selected for further investigations.

Effects of $\text{NO}_3^-/\text{NH}_4^+$ ratio

Muranaka et al (1992) reported that hairy roots of *Duboisia leichhardtii* cultured in modified Heller's medium containing 37 mM nitrate and no ammonium released scopolamine into the growth medium. Since B5-5 medium was suited for growth and alkaloid production, hyoscyamine and scopolamine release were examined in media containing five different nitrate/ammonium (N/A) ratios and compared with unmodified B5-5 medium (N/A ratio: 25/2), as shown in Fig. 3.

Transformed roots (50 mg fresh weight) were cultured in B5-5 medium for 4 weeks and then the alkaloid content in both roots and media were determined. Preliminary results (data not shown) demonstrated that in B5-5 medium prepared without NH_4^+ (N/A ratio: 25/0), the roots did not grow at all. Moreover, in the same medium supplemented with ammonium ions $[(\text{NH}_4)_2\text{HPO}_4]$ at a concentration greater than 10 mM (N/A ratio: <25/10), the hairy root growth was strongly inhibited. In comparison to the control, increasing the NH_4^+ concentration (N/A ratio: 25/5) decreased the alkaloid biosynthesis without modifying the hyoscyamine/scopolamine ratio. On the other hand, increasing the NO_3^- (KNO_3) concentration from 25 to 100 mM and keeping the NH_4^+ concentration constant dramatically enhanced the hyoscyamine content from 0.36% (dry weight) in the control to 0.54% (dry wt) in the medium with a N/A ratio of 100/2, but led to a nitrate-concentration-dependent inhibition of scopolamine. Thus, the hyoscyamine/scopolamine ratio increased significantly from 2.1 in the control medium to reach 4.8 in the modified B5-5 medium with a N/A ratio of 100/2. The addition of KNO_3 to the culture medium enhanced the hairy root growth remarkably. In medium supplemented with 75 mM KNO_3 , 353 mg (dry weight) were obtained after 4 weeks of culture. More KNO_3 (100 mM) strongly reduced the root growth which dropped to 198 mg (dry weight). As already reported by Muranaka et al. (1992), a high concentration

Table 1 Effect of feeding various precursors on the growth and hyoscyamine and scopolamine content of *Datura candida* × *D. aurea* hairy root cultures (FW fresh weight, DW dry weight, H/S hyoscyamine/scopolamine ratio)

Precursors	Concentration (mM)	Biomass (g FW/flask)	Alkaloid concentration (% DW)		Ratio H/S
			Hyoscyamine	Scopolamine	
None (control)	0	3.0	0.35	0.18	1.94
(R,S)-Phenyllactic acid	0.25	2.9	0.50	0.23	2.17
(R,S)-Phenyllactic acid	0.50	2.8	0.55	0.25	2.20
(R,S)-Phenyllactic acid/(R,S)-tropic acid	0.25/0.25	3.1	0.32	0.15	2.13
(R,S)-Phenyllactic acid/(R,S)-tropic acid	0.50/0.50	3.1	0.26	0.12	2.17
Ornithine	0.25	3.1	0.36	0.09	4.00
Ornithine	0.50	2.9	0.33	0.07	4.73

of NO_3^- in the form of KNO_3 may have some toxic effects on the physiological conditions of the root tissue. The increase in NO_3^- concentration also seems to have a direct inhibitory effect on the biosynthetic transformation of hyoscyamine to scopolamine. Furthermore, the roots submitted to various N/A ratios did not release any detectable tropane alkaloids into the culture medium, in contrast to the results of Muranaka et al. (1992) on the scopolamine release by *D. leichhardtii* hairy roots.

Effects of feeding metabolic precursors

The biosynthetic pathway for the formation of tropanes from the amino acids arginine/ornithine and phenylalanine is well documented (Leete 1990). More recently, it was demonstrated (Ansarin and Woolley 1993; Robins et al. 1994) that phenyllactic acid is an intermediate in the biosynthesis of the tropoyl moiety of hyoscyamine. However, the effects of feeding a number of potential intermediates to root cultures remain incompletely understood.

The effect of the addition of ornithine, an early precursor in the biosynthetic pathway, (*R,S*)-phenyllactic acid and (*R,S*)-tropic acid on hairy root growth and the accumulation of hyoscyamine and scopolamine is shown in Table 1. In all cases, the addition of precursors did not influence the biomass production which fluctuated between 2.8 and 3.1 g fresh weight/flask. Feeding (*R,S*)-phenyllactic acid led to a biosynthesis stimulation of both alkaloids. However, the hyoscyamine content was markedly stimulated whereas for scopolamine the increase was less important. The simultaneous feeding of (*R,S*)-phenyllactic acid and (*R,S*)-tropic acid at 0.25 mM each did not modify significantly the alkaloid concentration which is in contrast to the finding of Robins et al. (1994). At a higher level (0.50 mM), both hyoscyamine and scopolamine accumulation were severely inhibited to the same extent. When ornithine was fed, scopolamine biosynthesis was strongly inhibited even at 0.25 mM. On the other hand, there was no influence on hyoscyamine formation. Thus, the hyoscyamine/scopolamine ratio rose from 1.94 in the control to 4.00 and 4.73 in the presence of 0.25 and 0.50 mM ornithine, respectively.

Our results and those published in the literature suggest that the production of tropane alkaloids is not regu-

lated in a simple manner and that some enzymatic reactions may have limiting effects. In particular, the enzymes involved in early steps of the biosynthetic pathway are rate-limiting. Furthermore, the ratio of (*R,S*)-phenyllactic acid/(*R,S*)-tropic acid has some importance in controlling hyoscyamine and scopolamine production. Other studies are in progress to elucidate the rate-controlling metabolic fluxes and to develop a strategy for the production of tropane alkaloids on a larger scale. The kinetics of alkaloid release into the culture medium for continuous production of hyoscyamine and scopolamine are under investigation.

Acknowledgements The authors would like to thank Dr. W. C. Evans and Mr. J. Shaw (University of Nottingham) for supplying us with the seeds the *Datura* hybrid, Prof. P. Vuagnat for help performing the statistical analysis and Dr. L. Dinan who checked the English.

References

- Ansarin M, Woolley JG (1993) The obligatory role of phenyllactate in the biosynthesis of tropic acid. *Phytochemistry* 32: 1183–1187
- Christen P, Roberts MF, Phillipson JD, Evans WC (1989) High-yield production of tropane alkaloids by hairy root cultures of a *Datura candida* hybrid. *Plant Cell Rep* 8:75–77
- Christen P, Aoki T, Shimomura K (1992) Characteristics of growth and tropane alkaloid production in *Hyoscyamus albus* hairy roots transformed with *Agrobacterium rhizogenes* A4. *Plant Cell Rep* 11:597–600
- Dupraz J-M, Christen P, Kapetanidis I (1994) Tropane alkaloids in transformed roots of *Datura quercifolia*. *Planta Med* 60:158–162
- El-Dabbas SW, Evans WC (1982) Alkaloids of the genus *Datura*, section *Brugmansia*. X. Alkaloid content of *Datura* hybrids. *Planta Med* 44:184–185
- El-Imam YAM, Evans WC (1990) Alkaloids of a *Datura candida* cultivar, *D. aurea* and various hybrids. *Fitoterapia* 61:148–152
- Evans WC (1979) Tropane alkaloids of the Solanaceae. In: Hawkes JG, Lester RN, Skelding AD (eds) *The biology and taxonomy of the Solanaceae*. Academic Press, London, pp 241–254
- Gamborg OL (1970) Effects of amino acids and ammonium on the growth of plant cells in suspension cultures. *Plant Physiol* 45:372–375
- Geigy Scientific Tables (1982) 8th edn. Basle, vol 2, pp 209–210
- Leete E (1990) Recent developments in the biosynthesis of the tropane alkaloids. *Planta Med* 56:339–352
- Lloyd G, McCown B (1980) Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. *Int Plant Prop Soc Proc* 30:421–427

- Muranaka T, Ohkawa H, Yamada Y (1992) Scopolamine release into media by *Duboisia leichhardtii* hairy root clones. *Appl Microbiol Biotechnol* 37:554–559
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* 15:473–497
- Nussbaumer P (1996) Production d'hyoscyamine et de scopolamine par les racines transformées de *Datura candida* (Pers.) Saff. × *Datura aurea* (Lagerh.) Saff. (Solanaceae). Etude et développement d'un bioréacteur. Thesis, University of Geneva
- Ooms G, Karp A, Burrell MM, Twell D, Roberts J (1985) Genetic modification of potato development using Ri T-DNA. *Theor Appl Genet* 70:440–446
- Petit A, David C, Dahl GA, Ellis JG, Guyon P (1983) Further extension of the opine concept: plasmid in *Agrobacterium rhizogenes* cooperate for opine degradation. *Mol Gen Genet* 190:204–214
- Robins RJ, Parr AJ, Payne J, Walton NJ, Rhodes MJC (1990) Factors regulating tropane-alkaloid production in a transformed root culture of a *Datura candida* × *D. aurea* hybrid. *Planta* 181: 414–422
- Robins RJ, Woolley JG, Ansarin M, Eagles J, Goodfellow BJ (1994) Phenyllactic acid but not tropic acid is an intermediate in the biosynthesis of tropane alkaloids in *Datura* and *Brugmansia* transformed root cultures. *Planta* 194:86–94