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TROPINE ALKALOIDS PRODUCTION BY HAIRY ROOT CULTURES OF *DATURA STRAMONIUM* AND *HYOSCYAMUS NIGER*

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Key Word Index—*Datura stramonium*; *Hyoscyamus niger*; Solanaceae; *Agrobacterium rhizogenes*; hairy root culture; tropine alkaloids; hyoscyamine; scopolamine; TLC-densitometry.

Abstract—Hairy root were induced by inoculation of *Agrobacterium rhizogenes* in sterile seedlings of *Datura stramonium* and *Hyoscyamus niger*. The transformed roots cultures were established and subcultured on a Murashige and Skoog [1] hormone free medium. Several strains of hairy root were obtained from different genotypes of *D. stramonium*. Determination of hyoscyamine and scopolamine was performed by TLC-densitometry. A high variability of alkaloids production was observed between the different strains. Sucrose concentration as well as mineral composition of the culture medium influence the alkaloidal content of the transformed roots; scopolamine concentration expressed on a dry weight basis reached 0.56% in optimum conditions.

INTRODUCTION

Plant cell suspension cultures are an interesting alternative for secondary metabolites production [2]. In several studies, it was demonstrated that this production is higher in organ cultures (differentiated structures) than in dedifferentiated cells [3]; in any case a rapid biomass production is essential to get large scale metabolite production. However, it is assumed that nutritional and environmental requirements for cell proliferation and for production of secondary metabolites are generally different; for example, a two-stage culture method was developed for shikonine derivatives production in *Lithospermum erythrorhizon* cell cultures [4]. Transformed roots cultures, so called hairy root obtained after the insertion of T-DNA from root inducing (Ri) plasmid of *Agrobacterium rhizogenes* [5–8] into the plant genome, have both advantages of fast growth and organ differentiation. We report here the production of two tropine alkaloids of pharmaceutical importance by transformed roots of *Datura stramonium* and *Hyoscyamus niger*. The alkaloid biosynthesis by hairy root cultures of other Solanaceae species has been recently reported [9–12].

RESULTS AND DISCUSSION

Hairy root cultures were established from three different *D. stramonium* seedlings (D1, D2, D3). For one of them (D2), we isolated four different strains (D2a, D2b, D2c, D2d). All of them were obtained by the culture in the dark of the apex of a hairy root which appeared after inoculation. From *H. niger* only one strain was established (H1). As the synthesis of opines by hairy root cultures have a tendency to disappear after long term culture [8–10] or have been found to differ among individuals of the same clone [12], the confirmation of the transformed nature of the hairy root was grounded on the observation of three phenotypic features which distinguish them easily from ordinary root in culture: active proliferation, lack of geotropism and abundant lateral branching.

All results are summarized in Table 1. After 22 days of culture, a high but variable rate of biomass production was observed: from an initial inoculum of *ca* 1 g fresh weight the fresh weight of the transformed roots cultured in the same conditions (MS basal medium without hormone in the dark at 25°), increased up to 1.64 g fresh weight for (D3) strain and to 3.86 g fresh weight for (D2a) strain. All the strains were extracted and analysed for their alkaloidal content. As the production of hyoscyamine and scopolamine by callus and cells cultures of *D. stramonium* and *H. niger* has previously been reported and confirmed by HPLC, GLC and GS-MS [12, 13] and recently observed in hairy root cultures of *Atropa belladona* [10], the alkaloid identification was only performed by a specific and sensitive TLC method. The (H1) strain produces only hyoscyamine (0.07%) at a level comparable with that found in the plant growing *in vivo*. The different strains issued from *D. stramonium* produced no hyoscyamine but show marked differences in the content of scopolamine (from 0 to 0.56%). As observed in cell suspension cultures [14], hairy root expresses a biochemical variability between strains initiated from different plantlets (D1–D3) and between strains issued from the same plant (D2). The production of alkaloids was also affected by the mineral composition of the culture medium: on MS medium, the (D3) strain produce 0.11% of scopolamine and on Street medium the production was three times higher (0.32%). Higher sucrose concentrations (from 20 to 30 g/l) increases the scopolamine production in the (D1) strain (from 0.015 to 0.15%). Scopolamine was found nearly pure in the alkaloidal fraction of transformed roots strains of *D. stramonium*; alkaloid reagents showed only traces of other unidentified constituents on TLC plates. In the *H. niger* transformed roots extract, only hyoscyamine was detected. There was no evidence for the presence of alkaloids in the culture medium. In

Table 1. Alkaloid content of hairy root after 22 days of culture. (Initial inoculum 1 g fr. wt/flask)

Strain	Medium	Biomass production (g/flask) fresh weight	dry weight	Alkaloid content (% dry weight) Scopolamine	Hyoscyamine
D1	MS(30)	3.41	0.17	0.15	0
	MS(20)	2.09	0.06	0.015	0
D2 D2a	MS(30)	1.80	0.12	Trace	0
D2b	MS(30)	3.86	0.19	0.56	0
D2c	MS(30)	2.91	0.14	0.11	0
D2d	MS(30)	2.61	0.13	0	0
D3	MS(30)	1.64	0.06	0.11	0
	S(30)	1.47	0.05	0.32	0
H1	MS(30)	2.06	0.12	0	0.07

MS: Murashige and Skoog medium, S: Street medium; (X): concentration of sucrose (g/l) in the medium.

D: *D. stramonium.*

H: *H. niger.*

conclusion, these results prove that the use of the hairy root cultures from *D. stramonium* could be an interesting alternative route for the industrial extraction of scopolamine (high rate of biomass production and production of this alkaloid in a pure state). Using a selected high productive strain (D2b) optimized culture conditions [MS(30) medium and dark] and a one-stage cultivation method it was possible to increase both biomass rate and alkaloid production capacity.

EXPERIMENTAL

Bacterial strain. Agrobacterium rhizogenes ATCC15834 strain were grown on a solid medium containing yeast extract (1 g/l), peptone (5 g/l), sucrose (5 g/l) and $MgSO_4 \cdot 7H_2O$ (0.5 g/l); the pH was adjusted to 7.2.

Plant material. Seeds of *D. stramonium* and *H. niger* were surface sterilized (20 min) with $CaCl_2O_2$ soln (50 g/l.) then rinsed twice with sterile distilled H_2O. They were incubated on a mineral Street medium [11] in the dark at 25°.

Incubation. 10-day-old seedlings were infected with fast growing bacteria. The hypocotyls were wounded with a sterile needle containing bacteria. The inoculated plants were incubated at 25° under continuous light (2000 lx).

Establishment of hairy roots culture. After 3–4 weeks of incubation, terminal pieces of *ca* 20 mm long were excised from emerging hairy root and transfered to 1% agar MS medium without hormone but containing carbenicilline (1 g/l) to prevent bacterial contamination. After 3 days of incubation at 25° in darkness, apices of actively growing roots were transfered to a solid MS medium (stock cultures). For alkaloid production, transformed roots were cultivated in the dark on a liquid MS medium (15 ml) in 100 ml conical flasks on an orbital culture system.

Extraction and quantification of alkaloids. 22-day-old cultures were dried 48 hr at 40°. 1 g of dried transformed roots powder was mixed with 60 mg of $Ca(OH)_2$, 0.5 g of diatomaceous earth, 0.5 ml H_2O and allowed to stand for 15 min. This mixture was applied to a 8 mm i.d. glass column filled with diatomaceous earth (1 g). The column was eluted by 25 ml of peroxide-free Et_2O and the Et_2O fraction was extracted by 20 then 10 ml of 0.1 N H_2SO_4. After adjustment of the pH at 10 the combined solns were extracted first with 20 ml then with 10 ml of peroxide-free Et_2O; the Et_2O soln was filtered through dry Na_2SO_4 and evapd under red. pres. to dryness. The residue was dissolved in 0.5 ml MeOH and used for the TLC chromatographic identification and determination by comparison with standards of hyoscyamine and scopolamine. Silica gel $60F_{254}$ plates (10 × 20 cm) were used and developed with 1,1,1-$C_2H_3Cl_3$-$(C_2H_5)_2NH$ (9:1). After development and drying at 105° for 2 hr, the plates were sprayed with 4-dimethylaminobenzaldehyde (200 mg) dissolved just before use in 40 ml of EtOH–8N H_2SO_4 (1:1), then heated at 105° for 1 hr. The spots were measured with a TLC Scanner (Shimadzu CS-930) at 493 nm. The mean values were calculated from the integration of nine spots corresponding to three different concns of scopolamine and hyoscyamine and three spots of the soln of unknown concentration.

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