

# Hairy Root Technology: Effect of Etiolation on *Datura* sp. Transgenic Root Induction and Hyoscyamine Production

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## History

- Submission Date: 30-05-2019;
- Review completed: 09-06-2019;
- Accepted Date: 17-06-2019.

DOI : 10.5530/pj.2019.11.156

## Article Available online

<http://www.phcogj.com/v11/i5>

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## ABSTRACT

**Introduction:** Hyoscyamine, because of its medicinal properties, is an important tropane alkaloid. In order to implement a biotechnological process for its production, hairy roots of *Datura* species resulting from genetic transformation by *Agrobacterium rhizogenes* A4 strain have been the subject of this work. In the *in vitro* alkaloids production programs, optimization of the transformation is a crucial step for obtaining a large number of root lines allowing the selection of efficient lines. **Methods:** The conditions for hairy roots induction were explored by studying the effect of *Datura* sp. *in vitro* seedling etiolation on the genetic transformation. The first step was the establishment of *Datura in vitro* plantlet cultures followed by the hairy roots induction with A4 strain of *A. rhizogenes*. The confirmation of the genetic transformation was performed by polymerase chain reaction (PCR) analysis of *rolB* gene in the roots. After hyoscyamine extraction, it was analyzed (quantitatively and qualitatively) by gas chromatography. **Results:** 343 root lines were obtained in total, 96 root lines from non-etiolated *Datura in vitro* seedlings and 247 root lines from etiolated *in vitro* plantlets. After the selection of six transgenic root lines, two root lines from each species (*D. stramonium*, *D. tatula* and *D. innoxia*), one from non-etiolated *in vitro* plantlet and one from etiolated *in vitro* plantlet, the most hyoscyamine content was 8.43 mg/g D.W. obtained with *D. tatula* etiolated *in vitro* seedling. **Conclusion:** The etiolated *in vitro* plantlets seem more favorable to hairy roots induction.

**Key words:** *Datura* sp., etiolation, gas chromatography, hairy roots, hyoscyamine.

## INTRODUCTION

Some Solanaceae species including *Datura* genus biosynthesized in their roots alkaloids. The wild population of *Datura* species (*D. stramonium*, *D. tatula* and *D. innoxia*) that grows in Algeria is particularly rich in hyoscyamine. This last one is a tropane alkaloid used in medicine as useful natural product.<sup>1,2</sup>

*In vitro* plant cultures drawn an interest and cost-effective alternative to classical approaches to plant secondary metabolite production. They constitute sustainable and eco-friendly system to obtain complex chemical structures biosynthesized by concerned plant species. "Plant Cell Factory" concept defined as a biotechnological systems developed to improve the production of plant-derived compounds with biological activity.<sup>3</sup>

Among the plant *in vitro* production models of bioactive molecules those based on hairy roots techniques.<sup>4-6</sup> Following plant fresh wound, some soil bacteria are able to transfer some of their genetic information (plasmid) to the nuclear genome of the host plants that are sensitive. This plasmid DNA is then able to express itself. These bacteria are Gram- Rhizobiaceae of *Agrobacterium* genus. Among of them, *A. rhizogenes* responsible for hairy root (HR) disease.<sup>7</sup>

HR cultures characterized by a high growth rate and can synthesize secondary metabolites like as original plants roots. Naturally, root cultures

require an exogenous supply of plant hormones and have a low growth rate resulting in low biosynthesis of secondary metabolites. However, the use of HR culture has revolutionized plant tissue cultures for secondary metabolites biosynthesis. Their ease of maintenance and their ability to synthesize a wide range of chemical compounds offers an additional advantage as a continuous source of production.<sup>8,9</sup>

Maldonado-Mendoza *et al.*<sup>10</sup> have shown a great variability in the rate of growth and alkaloid content between the *Datura stramonium* root lines. This would result in considerable differences in root growth as well as in the accumulation of secondary metabolites even when they arise from the same transformation event.<sup>11,12</sup> Hence the interest, in a production program of secondary metabolites based on HR cultures, to induce the maximum number of root lines to increase the probability to select high-performing lines.

The aim of our work was the evaluating the effect of etiolation on hairy roots induction with *A. rhizogenes* in three *Datura* species. Our hypothesis was that etiolated *in vitro* seedlings are more sensitive to bacterial diseases than non-etiolated *in vitro* plantlets (exposed to light), transformation with agrobacteria would be more important (more transgenic roots induced on etiolated explants compared to explants from non-etiolated *in vitro* seedling). Knowing that root lines are unique, optimization of transformation by *A. rhizogenes* is an essential step for the selection of efficient hairy root.

**Cite this article:** HARFI B, KHELIFI L. Hairy Root Technology: Effect of Etiolation on *Datura* sp. Transgenic Root Induction and Hyoscyamine Production. Pharmacogn J. 2019;11(5):991-5.

## MATERIALS AND METHODS

### Establishment of *Datura* In Vitro plantlet cultures

Seeds of three *Datura* species (*D. stramonium* L., *D. tatula* L. and *D. innoxia* Mill.) collected in Mitidja (North of Algeria) constituted the vegetal material of this study. The seeds were scarified and surface disinfected according to Harfi *et al.*<sup>2</sup> protocol. They were sown in test tubes containing 20 ml of solid hormone-free Murashige and Skoog (MS) medium (Sigma, USA) containing 2% sucrose, 0.7% agar and pH adjusted at 5.6 – 5.8.

For each species, tow plots of 16 test tubes were constituted. The first plot represents the control group (non-etiolated *in vitro* plantlets) and were cultivated at 26 °C ± 1 °C with a daily 16 h-light photoperiod. The second one represents the group of etiolated *in vitro* plantlets and were cultivated at 26 °C ± 1 °C with total darkness. The experiment was repeated three times. For each species and for each culture conditions we used 48 test tubes. In total, 288 test tubes were used.

### Establishment of hairy root cultures

Hairy roots (HRs) cultures were obtained from hypocotyl fragments of 0.5 to 1 cm taken on the *Datura* sp. *in vitro* seedlings 45 days old via genetic transformation with *Agrobacterium rhizogenes* as described by Harfi *et al.*<sup>5</sup>. The used A4 strain of *A. rhizogenes* is an agropine-type plasmid. The co-culture (bacteria – explant) was done by simple deposit using a syringe on the level of the basal section of the hypocotyls. The culture of hypocotyls is operated on MS solid medium containing 250 mg / L cefotaxime. Only emergent roots from the infection site were retained. Root segments were excised when they reach approximately 2 cm length. HRs multiplication were carried out on fresh B5 solid medium without antibiotic and were cut off in 2 cm long segments.

The effect of seedling etiolation on HRs induction was measured according the three main following criteria:

- Time of appearance of the first root (day);
- Average number of roots per explant;
- Total obtained root (from 48 explants) per species.

### Confirmation of transformation and selection of efficient root lines

Genetic transformation with A4 strain of *A. rhizogenes* was confirmed by the presence of *rol* genes (*rolB*) in HRs using PCR (Polymerase Chain Reaction).<sup>2</sup>

The selection of the efficient root lines was based on two main criteria:

- HR biomass (mg / 50 ml of B5 liquid medium after 20 days of culture);
- Hyoscyamine content (mg / g D.W.).

### Tropane alkaloids extraction

Hyoscyamine extraction was carried out according to Amdoun *et al.*<sup>13</sup> protocol. HRs samples of 50 mg dried for 48 h at 40 °C and powdered,

tropane alkaloids were extracted with HCl 0.1 N solution. NH<sub>4</sub>OH was used to reach pH 10. Aqueous phase was filtered and extracted three times with an equal volume of chloroform. The organic phase was evaporated and the residue was suspended in dichloromethane.

### Gas chromatography analysis

Gas chromatography (GC) was performed on a CHROMPACK CP 9002 (Chrompack International B.V., Middelburg, The Netherlands), equipped with a flame ionization detector (FID). A fractionated sample of 2 µL was injected into a capillary DB1 column. The used GC conditions were as described by Harfi *et al.*<sup>5</sup> (carrier gas: nitrogen; Combustion gas: hydrogen; detector temperature: 260 °C; injection temperature: 260°C and oven temperature: 250 °C). Hyoscyamine identification and quantification were performed using a standard prepared under the same conditions as the samples. Each sample analysis was repeated in triplicate.

### Statistical analysis

After data homogeneity and normality tests, statistical analysis was performed. It was carried out with SPSS software version 20.0 according to variance analysis (one-way ANOVA) and Tukey's test for the obtained results. The values followed by the same letter are not significantly different from each other (P 0.05: level of significance mean).

## RESULTS

The interest of our work was to evaluate the effect of *in vitro* seedlings etiolation on hairy roots induction of three *Datura* species using *A. rhizogenes*. The aim was to improve the number of induced root lines in order to increase the probability of selecting efficient lines in terms of hyoscyamine and biomass productions. Figure 1 illustrates the experimental protocol steps adopted from the establishment of *in vitro* seedlings (etiolated and non-etiolated) until the selection of efficient root lines.

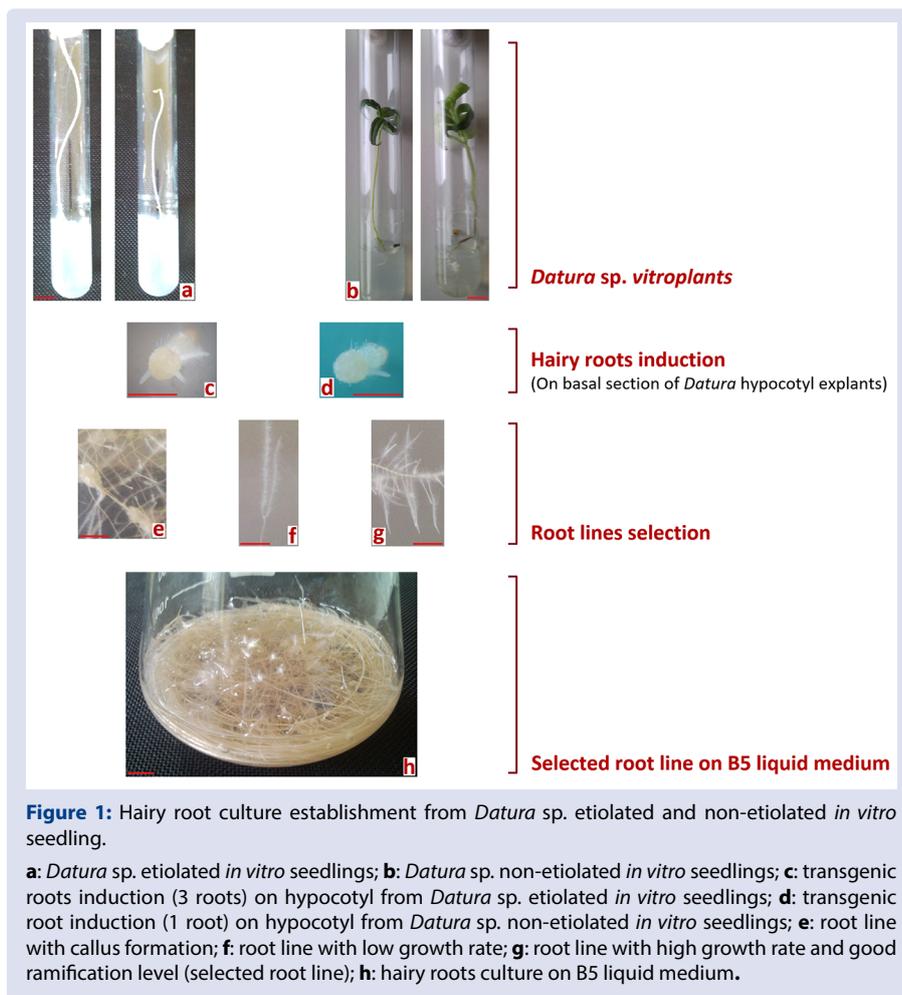
The statistical study reveals a significant effect of seedling etiolation on hairy roots induction (Table 1). For the mean time of appearance of the first root, the most interesting homogeneous group (a) is represented by etiolated *in vitro* seedlings of *D. tatula* explants with a mean time of 7.92 days. It was with the explants of *D. stramonium* etiolated seedlings that we recorded the statistically highest average number of transgenic roots per explant (2.62). Also, the total number of root lines obtained from 48 explants was greater in etiolated seedlings than in non-etiolated *in vitro* seedlings. There were 126, 93 and 28 root lines respectively for the explants of the etiolated *in vitro* seedlings of *D. stramonium*, *D. tatula* and *D. innoxia* and 54, 31 and 11 root lines respectively for the explants of non-etiolated *in vitro* seedlings of *D. stramonium*, *D. tatula* and *D. innoxia*. Explants from etiolated *in vitro* plantlets show the best genetic transformation capacities compared to non-etiolated explants.

On the whole of all obtained root lines from the three *Datura* species (343 lines in total, 96 root lines from non-etiolated *in vitro* seedlings and 247 root lines from etiolated *in vitro* plantlets), six root lines were retained on the basis of their hyoscyamine content (Table 2). For each species, two root lines were retained, one from the etiolated *in vitro* seedlings and one from the non-etiolated *in vitro* plantlets.

**Table 1: Effect of *in vitro* seedlings etiolation on *Datura* sp. hairy roots induction.**

	Non-etiolated <i>in vitro</i> seedling			Etiolated <i>in vitro</i> seedling		
	DS	DT	DI	DS	DT	DI
Time of appearance of the first root (d)	16.58 <sup>c</sup>	15.24 <sup>c</sup>	16.97 <sup>c</sup>	8.75 <sup>a</sup>	7.92 <sup>a</sup>	11.02 <sup>b</sup>
Average number of roots per explant	1.12 <sup>C</sup>	0.64 <sup>D</sup>	0.23 <sup>E</sup>	2.62 <sup>A</sup>	1.94 <sup>B</sup>	0.58 <sup>D</sup>
Total obtained root (from 48 explants)	54	31	11	126	93	28

The values are given as the mean ± SD, different letters indicate significant difference between values according to ANOVA test (p: 0.05); d: days; DS: *Datura stramonium in vitro* seedlings; DT: *D. tatula in vitro* seedlings; DI: *D. innoxia in vitro* seedlings.



**Figure 1:** Hairy root culture establishment from *Datura* sp. etiolated and non-etiolated *in vitro* seedling.

**a:** *Datura* sp. etiolated *in vitro* seedlings; **b:** *Datura* sp. non-etiolated *in vitro* seedlings; **c:** transgenic roots induction (3 roots) on hypocotyl from *Datura* sp. etiolated *in vitro* seedlings; **d:** transgenic root induction (1 root) on hypocotyl from *Datura* sp. non-etiolated *in vitro* seedlings; **e:** root line with callus formation; **f:** root line with low growth rate; **g:** root line with high growth rate and good ramification level (selected root line); **h:** hairy roots culture on B5 liquid medium.

**Table 2: Hyoscyamine content of selected root lines from non-etiolated / etiolated *Datura* sp. *in vitro* seedlings.**

<i>Datura</i> sp. <i>in vitro</i> seedling	Selected root lines	Hyoscyamine content (mg/g D.W.)
Non-etiolated	NE-L <sub>DS</sub>	8.18 ± 0.54 <sup>a</sup>
	NE-L <sub>DT</sub>	6.06 ± 0.38 <sup>b</sup>
	NE-L <sub>DI</sub>	2.01 ± 0.20 <sup>d</sup>
Etiolated	E-L <sub>DS</sub>	8.21 ± 0.72 <sup>a</sup>
	E-L <sub>DT</sub>	8.43 ± 0.42 <sup>a</sup>
	E-L <sub>DI</sub>	3.04 ± 0.27 <sup>c</sup>

The values are given as the mean ± SD, different letters indicate significant difference between values according to ANOVA test (p: 0.05); D.W.: Dry weight; NE-L<sub>DS</sub>: Selected root line from non-etiolated *Datura stramonium in vitro* seedlings; NE-L<sub>DT</sub>: Selected root line from non-etiolated *D. tatula in vitro* seedlings; NE-L<sub>DI</sub>: Selected root line from non-etiolated *D. innoxia in vitro* seedlings; E-L<sub>DS</sub>: Selected root line from etiolated *D. stramonium in vitro* seedlings; E-L<sub>DT</sub>: Selected root line from etiolated *D. tatula in vitro* seedlings; E-L<sub>DI</sub>: Selected root line from etiolated *D. innoxia in vitro* seedlings.

The statistical study of etiolation on hyoscyamine content of the six retained root lines reveals a significant effect of the etiolation on the tropane alkaloid content for *D. tatula* and *D. innoxia* species. However, for the *D. stramonium*, etiolation of *in vitro* plantlets did not affect the hyoscyamine content. This may be explained by the number of induced root lines with non-etiolated *D. stramonium* explants that is relatively high (54 lines), which has made it possible to select a root line with a high hyoscyamine content.

## DISCUSSION

The development of genetically transformed plant tissue cultures and mainly HRs induced by *A. rhizogenes* is a key step in the use of *in vitro* cultures for the production of secondary metabolites. HRs can be a promising source for standardized production of secondary

metabolites. It is generally admitted that HRs produce secondary metabolites generation after generation without loss of genetic and biosynthetic stabilities.<sup>14,15</sup>

The etiolated *in vitro* seedlings are characterized by an absence of chlorophyll in the tissues and an excessive elongation of the plantlets. Light is a key factor for the growth, development and response to plant stress. The plant cell is equipped with "light-signal" mechanisms located within the chloroplast that is involved in the defense of the plant. Light perception and stress response could work and act together to form a plant defense network.<sup>16</sup> According to Yaeno *et al.*<sup>17</sup> trienoic fatty acids in chloroplast lipid membrane are decisive for adaptation to stress and play an important role in the regulation of the plant's defense against external aggressions (biotic and abiotic stresses). In addition,

the reinforcement of structural barriers with lignin and suberin at the cellular level is also a common response in plants exposed to ultraviolet light.<sup>18</sup>

Indeed, the improvement of the results of the HRs induction by *A. rhizogenes* with the explants from etiolated *in vitro* seedlings can be explained by the fragility of etiolated plantlets, they were more susceptible to microbial attack than non-etiolated ones. This sensitivity would induce a more favorable response to the transformation by the *A. rhizogenes*.

In the spontaneous whole plants of *Datura* species the mean hyoscyamine contents are between 0.2 – 0.8 mg/g D.W.<sup>1</sup>. Also, the average hyoscyamine levels of the HRs of these species are 2.1 mg/g D.W. and 1.7 mg/g D.W. respectively for *D. stramonium*<sup>12</sup> and *D. innoxia*<sup>19</sup>. The best hyoscyamine contents obtained in our study were 8.21 mg/g D.W. for selected root line from *D. stramonium*, 8.43 mg/g D.W. for selected root line from *D. tatula* and 3.04 mg / g D.W. for selected root line from *D. innoxia*. Our results compared to those cited in the bibliography show the interest of inducing a maximum of transgenic root lines in order to select lines with an important hyoscyamine contents. According to Zhi-Bi and Min<sup>11</sup> and Georgiev *et al.*<sup>12</sup>, each root line obtained following transformation by *A. rhizogenes* can be considered a different line from all others on a genetic and biochemical level. Thus, in the production programs of alkaloids via *in vitro* plant culture technics, the optimization of the transformation by the *A. rhizogenes* is a crucial and unavoidable step for obtaining a large number of HRs allowing the selection of efficient lines.

## CONCLUSION

HRs cultures are characterized by a high growth rate and can synthesize secondary metabolites in the same way as the roots of the original plants. Naturally, root cultures require an exogenous supply of plant hormones and have a very slow growth rate resulting in low secondary metabolites synthesis. However, the use of HR cultures has revolutionized the plant tissues cultures for the biosynthesis of secondary metabolites. Their ease maintenance and their ability to synthesize a wide range of chemical compounds offers an additional advantage as a continuous source of production of secondary metabolites.<sup>8,9</sup>

From all obtained results, we confirmed our initial hypothesis “*etiolated in vitro seedlings are more sensitive to hairy root disease than non-etiolated in vitro plantlets*”. Our study confirms the interest of improving root lines induction using genetic transformation with *A. rhizogenes* in program drawn up on hairy root technology for a plant secondary metabolites production.

## ACKNOWLEDGEMENTS

This work has been carried out at the Laboratory of Genetic Resources and Biotechnology (L-RGB) of the National Higher School of Agronomy (ENSA – El-Harrach), Algiers, ALGERIA.

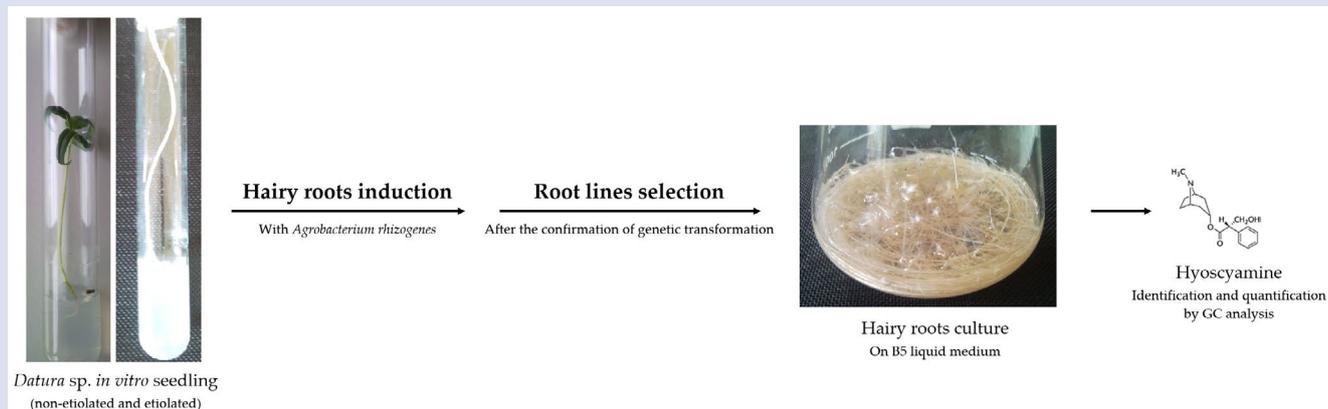
## CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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## GRAPHICAL ABSTRACT



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**Cite this article:** HARFI B, KHELIFI L. Hairy Root Technology: Effect of Etiolation on *Datura* sp. Transgenic Root Induction and Hyoscyamine Production. *Pharmacog J.* 2019;11(5):991-5.