

Transgenic Plants from Ri-Transformed Callus of *Datura innoxia*. Alkaloid Content of Normal and Transformed Tissues

H. Elhag, A. Gohar, M.M. El-Olemy and J.S. Mossa

Department of Pharmacognosy, College of Pharmacy,
King Saud University,
P.O. Box 2457, Riyadh 11451, Saudi Arabia

ABSTRACT. Hairy roots were induced by inoculation of sterile plantlets of *Datura innoxia* with *Agrobacterium rhizogenes*. The hairy roots were separated and maintained on hormone-free liquid MS medium. They proliferated 30 fold, based on the initial fresh weight, after 4 weeks of subculturing. Some segments of the hairy roots proliferated into undifferentiated callus (transgenic callus), which in turn differentiated into transgenic shoots, upon transfer to the light on the same basal MS medium. The shoots were rooted by subculturing on either hormone-free medium or on MS medium supplemented with 0.3 mg/l IAA to yield transgenic plantlets. Genetic transformation into hairy roots, transgenic callus and plantlets was confirmed by chromatographic detection of opines in these tissues. The hairy roots gave the highest total alkaloids and hyoscyamine contents and was second to the normal plant in scopolamine production. The alkaloid content of the normal plant was higher than that of the tested transgenic plants, transgenic callus and normal callus.

Hairy roots (HR) have been successfully induced from a number of solanaceous species known to produce the pharmacologically valuable compounds hyoscyamine and scopolamine and were shown to synthesize such alkaloids (Flores *et al.* 1987, Saito *et al.* 1992, Hashimoto *et al.* 1993, Verpoorte *et al.* 1993 and O'Keefe and Beecher 1994). These HR could be grown in small fermenters as potential systems for commercial production of such tropane alkaloids (Hashimoto *et al.* 1993). Over fifteen *Datura* species have been successfully transformed with various strains of *Agrobacterium rhizogenes* (Saito *et al.* 1992).

Since scopolamine is more valuable than hyoscyamine, as it produces less undesirable CNS effects, extensive studies are being made to improve the commercial production of scopolamine. Hairy roots of scopolamine-producing plants may be appropriate systems for such a purpose.

Transgenic plants have been reported from HR of certain *Atropa* and *Nicotiana* species (Saito *et al.* 1992). However, nothing is reported regarding such transgenic plants from HR of *Datura* species.

The purpose of the present investigation was to induce HR from *D. innoxia*, and to develop transgenic callus and plants thereof. The ability of such tissues to accumulate the tropane alkaloids hyoscyamine and scopolamine was investigated, in comparison to normal plants and callus.

Experimental

Initiation of hairy roots. Hairy roots were initiated on sterile axenic plantlets of *D. innoxia* (10 cm long and 21 days old) by infection with *Agrobacterium rhizogenes* strain # 15834, maintained on YMB agar medium (Ooms *et al.* 1985). Hairy roots developed on the infected plantlets (leaves and stems) within 3-4 weeks; these were separated and cultured on hormone-free solid MS medium, containing 0.75 g/l cefuroxime (Glaxo, England) for two successive subcultures. The bacteria-free HR were maintained by continuous subculture onto fresh hormone-free MS medium every 4 weeks in the dark at 23°C. For liquid cultures, 1 g of HR was inoculated into 100 ml medium/500 ml-flask and kept on a rotary shaker at 100 rpm in the light (16 hr photoperiod, 22.5 $\mu\text{mol s}^{-1}\text{m}^{-2}$ from cool-white fluorescent lamps).

Production of transgenic callus and plantlets. Hairy roots grown on solid MS medium in the dark, spontaneously produced callus which continued to grow while also producing HR. This transgenic callus was maintained by continuous subculture every 4 weeks in the dark at 23°C. Upon transfer of this callus to the light, some clumps turned green and differentiated into shoots. Such shoots were rooted on MS medium supplemented with 0.3 mg/l IAA or on 1/2 MS medium. Rooted plantlets were transferred to soil in the greenhouse (transgenic plants).

The established HR, transgenic callus and regenerated transgenic plants were subjected to analysis of opines and alkaloids in comparison to the normal callus and plants.

Detection of opines. Fresh transformed tissues, HR, transformed callus and leaves of transformed plants, 0.5 gm each, were separately triturated with 1 ml

water, centrifuged and the aqueous solution was examined by paper chromatography on Whatmann #1/n-BuOH-AcOH-H₂O (4:1:5); 3 runs; detection was done with alkaline AgNO₃ spray, followed by heating at 110°C for 10 min. (Kamada *et al.* 1986). Reference agropine and mannopine as well as aqueous extracts of normal plants and callus were also spotted for comparison.

Isolation of the alkaloidal fractions. The freeze-dried plant material, 1 gm each, was extracted (cold maceration) with CHCl₃-CH₃OH-NH₄OH (15:5:1), three times with 50, 25 and finally 25 ml. The alkaloidal fraction, in each case, was isolated from the obtained extract by acid-base purification (Anonymous 1985). The final alkaloidal CHCl₃ extract of each was adjusted to 50 ml in a volumetric flask. Appropriate volumes of the extracts, accurately measured, were used for the titrimetric determination of the contained alkaloids, as well as TLC and GLC analysis.

Determination of the total alkaloids. Quantification of the total alkaloids was done according to the B.P. non-aqueous titrimetric method (Anonymous 1980). A volume of 20 ml of the alkaloidal CHCl₃ extract of each sample was used for such determination.

TLC Analysis. An aliquot of the alkaloidal fraction of each sample was concentrated and chromatographed, against authentic tropanes (Table 1), using silica gel 60 F 254 plates/CH₃OH-NH₄OH (100:1.5).

Table 1. TLC and GC Identification of alkaloids of the investigated tissues and semiquantitative estimation of amounts present

Spot No.	TLC R _f	GC* R _f /min	Investigated tissues					Identification
			1	2	3	4	5	
1	0.88	-	+	+	++	+	+	unknown
2	0.81	18.45	++	+	++	+++	+	scopolamine
3	0.67	-	-	-	++	+	-	unknown
4	0.57	16.70	±	±	+++	++	+	hyoscyamine
5	0.41	-	-	-	++	-	-	unknown
6	0.37	2.44	-	-	+	-	-	tropine
7	0.29	4.13	-	-	+	-	-	scopine

*Silylated derivatives.

1 Normal callus.

2 Transgenic callus.

3 Hairy roots.

4 Normal plant.

5 Transgenic plant.

Table 2. Alkaloid production in *D. innoxia* hairy roots and normal (N) and transgenic (T) callus and plants*

Sample	Total alkaloids** mg/g	Hyoscyamine*** mg/g	Scopolamine*** mg/g
N. callus	1.24	0.11	1.03
T. callus	1.71	0.11	0.61
H. roots	7.49	1.30	1.03
N. plant	5.76	1.01	2.03
T. plant	3.53	0.40	0.87

* Expressed as mg/g dry weight.

** Total alkaloids determination by titrimetric method.

*** Hyoscyamine and scopolamine determination by GC method.

GLC Analysis. A volume of 5 ml of the alkaloidal CHCl_3 extract, was evaporated to dryness (40°C). The residue was silylated with Tri-Sil/BSA (Pierce Chem. Co., USA) at R.T. for 5 min., and the final volume was adjusted to 5 ml and subjected to GC analysis (Varian 3700 GC with FID): GLC column (2m x 2mm): 3% OV-17 on Chromosorb W (80-100 mesh); carrier gas N_2 ; flow rate : 50 ml/min; temperature program: 130-280°C at a rate of 8°C/min, then isothermal at 280°C for 4 minutes; volume injected: 1 μL . The contents of both scopolamine and hyoscyamine were determined from the appropriate standard curve (Table 2).

Results and Discussion

Hairy roots of *D. innoxia* were developed using a virulent strain of *Agrobacterium rhizogenes* (strain 15834), harboring Ri plasmid (PR; 15834). Such HR grew well in liquid hormone-free MS medium, giving 30 times its weight in four weeks and tested positive for opines. When grown on hormone-free solid MS medium in the dark, some of the HR spontaneously developed undifferentiated callus. This callus continued to grow, while also producing occasional HR. Such mutual production of HR and callus continued for several subcultures. The transgenic nature of this callus was confirmed by the detection of opines.

When grown on solid hormone-free MS medium, in the light, the transgenic callus developed green clumps or aggregates which eventually formed shoots. Such shoots were rooted either on MS solid medium containing 0.3 mg/l IAA or on 1/2 MS medium; occasionally, they developed further shoots or formed callus at their

bases. The rooted plantlets were finally transferred to the greenhouse and their transgenic nature was confirmed by the detection of opines in the fresh tissues. Both agropine and mannopine were detected in the H.R., transgenic callus and transgenic plants.

Concerning alkaloid production (Table 2), the hairy roots gave the highest content of the both total alkaloids (7.49 mg/g) and hyoscyamine (1.30 mg/g). These results are in general agreement with the earlier studies on *Datura* species and other solanaceous plants (Kamada *et al.* 1986, Payne *et al.* 1987, Jaziri *et al.* 1988 and Shimomura *et al.* 1991). Transgenic plants were lower in their total alkaloids, hyoscyamine and scopolamine production than the normal plants. This could be explained through an altered control of the biosynthetic pathways brought about by the introduced foreign genes of the *A. rhizogenes* (O'Keefe and Beecher 1994); or that the enzyme(s) coded by such genes, may catalyse the conversion of the needed intermediate metabolites into other secondary constituents. Normal callus was lowest in the total alkaloidal content, although it produced much higher scopolamine than hyoscyamine. Transgenic callus gave lower scopolamine than normal callus. This transgenic callus, however, when grown in the light (solid medium), showed higher total alkaloids and scopolamine content than when grown in the dark (1.95 mg and 0.90 mg/g dry weight, respectively). Light thus induced higher conversion of hyoscyamine into scopolamine as well as increased total alkaloid synthesis. However, such system would not be suitable for commercial exploitation, since this was done in solid (callus) culture. It thus appears that the best system for production of both hyoscyamine and scopolamine using *D. innoxia*, is the hairy root culture or the normal plants. Because of the fast growth rate of the HR and the suitability of growing them in industrial scale fermenters (Hashimoto *et al.* 1993), the hairy roots, among *D. innoxia* tissues tested, appears to be the most suitable system for commercial production of hyoscyamine and scopolamine.

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استنباط نباتات متحولة وراثيا من أنسجة (كذب) نبات *Datura innoxia* بواسطة بكتريا الجذور

حامد الحاج و أحمد جوهر و محمود العليمي و جابر سالم موسى

قسم العقاقير - كلية الصيدلة - جامعة الملك سعود

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تم استنباط جذور شعرية Hairy roots عن طريق إصابة أوراق نبات *Datura innoxia* خال من الامراض ببكتريا الجذور *Agrobacterium rhizogenes* فصيلة ١٥٨٣٤ وقد تم التخلص من الاجروبيكتريا من تلك الجذور الشعرية بزراعتها على وسط غذائي MS يحتوي على مضاد حيوي Cefuroxime لثلاثة اجيال . ومن ثم تكاثرت الجذور الشعرية الخالية من البكتريا على وسط غذائي سائل Liquid MS خال من منظمات النمو واعطت معدل نمو ثلاثون ضعفاً بالمقارنة لوزنها الأصلي بعد شهر واحد . وعند نقل تلك الجذور على وسط غذائي صلب Solid MS في الظلام فان تلك الجذور الشعرية تكون أنسجة كذب غير متشكلة undifferentiated callus وعند نقل هذا الكذب للضوء على نفس الوسط الغذائي فان بعض الخلايا تتجمع وتكتسب اللون الاخضر وتشكل إلى ان اعطت نبيتات لا جذرية shoots وعند نقل تلك النبيتات اللاجذرية إلى وسط MS يحتوي على ٠,٣ مجم بالتر من أندول حمض الخليك Indoleacetic acid فانها تكون جذوراً لتصبح نبيتات كاملة (نباتات متحولة Transgenic plants) تم نقلها إلى التربة . وقد تم اثبات التحول الوراثي

Transgenic nature في الجذور الشعرية وفي الكذب المتحول (Transgenic callus) وفي النباتات المتحولة بالتعرف على الاحماض الامينية الخاصة ببكتريا الجذور (opines) في تلك الأنسجة بواسطة كروماتوجرافيا الورق واستخدام مواد مرجعية من تلك الاحماض . . في حين ان النبات العادي والانسجة غير المتحولة كانت خالية من تلك الاحماض . . مما يدل ان هناك تحولا وراثيا قد تم بالفعل عن طريق اندماج الجين الخاص ببكتريا الجذور (Ri gene) مع جينات تلك النباتات والانسجة المتحولة . . وبدراسة محتوى القلوانيات بتلك الانسجة (الجذور الشعرية والكذب المتحول والنباتات المتحولة) بالمقارنة للكذب العادي والنباتات العادية (غير المتحولة) فقد ثبت احتواء الجذور الشعرية على أعلى نسبة من القلوانيات الكلية والهيسيامين ، اما النباتات العادية (غير المتحولة) فكانت أقل من محتوى القلوانيات الكلية من الجذور الشعرية ولكنها كانت اعلى منها في محتواها من السكوبولامين Scopolamine وكانت في محتواها من القلوانيات الكلية ومن الهيسيامين أعلى من النباتات المتحولة . . اما الانسجة غير المتشكلة (الكذب) فقد احتوت على أقل نسبة من القلوانيات وبخاصة انسجة الكذب المتحولة .