Enhancement of tropane alkaloids production in Egyptian henbane (*Hyoscyamus muticus* L.) *via Agrobacterium rhizogenes* transformation

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Raoufa A. Abdel Rahman* and H.S. Taha **

*-Biopharmacutical Products Research Department, Mubarak City for Scientific Research and Technology Applications, Borg El-Arab, Alexandria, Egypt.

**-Plant Biotechnology Department, National Research Centre, Dokki, Cairo, Egypt.

** To whom correspondence should be addressed. E-mail: Hussein03@yahoo.com

ABSTRACT

An efficient protocol for establishment of transgenic Egyptian henbane (Hyoscyamus muticus L.) root cultures using Agrobacterium rhizogenes is reported. Two strains of A. rhizogenes 15834 and A4, carrying the pBI121 binary vector, were tested for their ability to produce hairy roots on wounded hypocotyl, leaf and stem explants as well as complete seedlings. One of the strains induced hairy root formation on all explants, whereas the other type caused the growth of tumorigenic calli or produced no response. The effectiveness of different concentrations of Kanamycin as antibiotic for selective medium of hairy root production was investigated. On other hand, the effect of transgenic hairy root cultures on enhancement of total tropane alkaloids, hyoscine and hyoscyamine production was investigated as compared with the production of tropane alkaloids from different calli cultures obtained.

Key words: *Hyoscyamus muticus L. Agrobacterium rhizogenes, tropane alkaloids, HPLC.*

INTRODUCTION

Secondary metabolites are low-molecular-weight compounds produced widely throughout the plant kingdom. Plant alkaloids constitute the largest groups of natural products, providing many pharmacologically active compounds. The in-depth understanding of biosynthetic pathways, along with the increasing number of cloned genes involved in biosynthesis, enable the exploration of metabolic engineering as a potential effective approach to increase the yield of specific metabolites by enhancing rate-limiting steps or by blocking competitive pathways. A few genera of the plant family *Solanaceae*, including *Hyoscyamus, Duboisia, Atropa*, and *Scopolia* are able to produce biologically active nicotine and tropane alkaloids simultaneously (Caldentey *et al*., 2004).

Hyoscyamus muticus L., the Egyptian henbane is one of the most important medicinal plants and has been utilized in traditional medicine as a sedative, anodyne, calmative and antispasmodics. Also, it is similar to atropine in its ability to dilate the pupil of the eye (Strauss, 1989).

Scopolamine (hyoscine) is a pharmaceutically important tropane alkaloid extensively used as an anticholinergic agent (Zhang *et al*., 2004).

Transgenic hairy root cultures have served as a useful model system to investigate the biosynthesis of alkaloids, and a variety of other secondary metabolites. Therefore, transformed root cultures derived from members of the Solanaceae have been used extensively to study the production of tropane alkaloids and nicotine (Hamill *et al.*, 1990, Robins *et al.*, 1991, Hashimoto *et al.*, 1993, Sharp and Doran, 1999 and Aoki *et al.*, 2001). The *Agrobacterium*-mediated production of hairy roots also creates a rapid and simple means to introduce and express foreign genes in plant cells that are capable of synthesizing specific secondary metabolites (Jouhikainen *et al.*, 2003).In this investigation, the development of an efficient protocol to introduce foreign genes into transgenic Egyptian henbane (*H. muticus* L.) hairy root cultures using *A. rhizogenes* is described. Rapidly growing transformed hairy root cultures could serve as a simple, reliable and well-defined model system to study the molecular regulation of genes encoding tropane alkaloid biosynthetic enzymes, and to evaluate the potentiality to metabolically engineer these important medicinal plants.

MATERIALS AND METHODS

Seed sterilization and germination

Seeds of *Hyoscyamus muticus* L. were surface-sterilized with 70% (v/v) ethanol for 30 sec and 20% (v/v) commercial Clorox solution for 10 min, then rinsed three times in sterilized water. Approximately 30 seeds were placed on 25 ml of agar-solidified culture medium in glass jars 250 ml. The basal Murashige and Skoog (1962) medium was solidified with agar at 0.6 % (w/v). The medium was adjusted to pH 5.8 before adding agar and then sterilized by autoclaving at 1.2 kg cm^{-2} (121°C) for 15 min. The seeds were germinated in a growth chamber at 25 °C under standard cool white fluorescent tubes with a flux rate of 35 µmol s^{-1} m^{-2} and a 16 hr photoperiod.

Callus production

Obtained calli tissue from hypocotyl, leaf and stem explants as well as complete seedlings of *H. muticus* were induced according the method described by Taha *et al.* (2002).

Preparation of *Agrobacterium rhizogenes*

The binary vector pBI121 (Jefferson *et al.*, 1987) was mobilized in *Agrobacterium rhizogenes* strains 15834 and A4. Transformed *A. rhizogenes* cultures were grown to mid-log phase (A_{600}=0.5) at 28 °C on a gyratory shaker at 180 rpm in liquid Luria-Bertani Medium (LBM medium) (1% [w/v] tryptone, 0.5% [w/v] yeast extract, and 1% [w/v] NaCl, pH 7.0), containing 50 mg l^{-1} Kanamycin. The bacterial cells were collected by centrifugation for 10 min at 1500 rpm, and resuspended at a cell density of A_{600}=1.0 in liquid inoculation medium Gamborge *et al.* (1968) (B_5) salts and vitamins medium containing 20 g l^{-1} sucrose).

Production of transgenic hairy root cultures

Excised hypocotyl, leaf, stem explants and complete seedlings after 10 days old of seed germination of *Hyoscyamus muticus* L, were used as the explant materials for co-cultivation with *A. rhizogenes*. Various explants and complete seedlings were randomly wounded using a scalpel, and then immersed in an *A. rhizogenes* culture suspended in liquid inoculation medium for 10–15 min, blotted dry on sterile filter paper, and incubated in the dark on agar-solidified MS-medium. After 2 days of co-cultivation, different explants were transferred to hormone-free selection media mainly containing MS-salts and vitamins, 3% (w/v) sucrose. Different

concentrations of the antibiotic kanamycin 50 mg l^{-1}, 100 mg l^{-1}, 150 mg l^{-1}, 200 mg l^{-1} and 250mg l^{-1} were tested. Agar at 8 g l^{-1} was used as a gelling agent. Within 4–5 weeks, numerous kanamycin-resistant roots had emerged from the wound sites. The hairy roots were separated from the derived explants and sub-cultured in darkness at 25°C on hormone-free selection medium. After repeated transfer to fresh selection medium, rapidly growing hairy root cultures were obtained. Growth rates were determined by measuring the fresh weight of cultured roots for five weeks (interval 1-week). The addition of various concentrations of the auxins analogues indole-3-acetic acid (IAA), indole-3-butyric acid (IBA) and 1-naphthaleneacetic acid (NAA) to the culture medium was tested to promote the growth of hairy roots. All experiments were conducted in three replicates and repeated at least twice.

Extraction and determination of tropane alkaloids using *HPLC* analysis

According to the method described by British Pharmacopoeia (1998), the extraction of total alkaloids, hyoscine (scopolamine) or hyoscyamine in the transformed hairy roots or obtained calli cultures from different explants of *H.muticus* were performed. However, quantitative tropane alkaloid (hyoscyamine and hyoscine) determinations had been estimated according to Milan *et al.* (1990).

Chromatographic conditions

Apparatus:- Beckman, pump: 126 solvent module, diode array: 168 detector, auto sampler: 50 TE Auto sampler, soft wear : system gold Column: hyper clone SUODS (C18), 250x4.6mmx5 micron+grad column 5 cm, mobile phase: acetonitrile: 30 % (1 L H_2O + 3 ml Triethanol amine TEA) 70 % , temperature: 35 °C, run time : 25 min, wave length: 254 nm Flow: 1 min/min.

RESULTS

Establishment of hairy root cultures

Two different strains of *A. rhizogenes* 15834 and A4 were tested for their ability to induce the formation of hairy roots on different explants and complete seedlings of *H. muticus* L. Wounded *Hyoscyamus muticus* L. explants and the complete seedlings were highly susceptible to infection by the strains of *A. rhizogenes* as shown by the explants emerged from kanamycin-resistant tissues. As shown in Table (1), the strain A4 infected more than 90 % of the explants and induced an average of three to four hairy root initials per explant or seedling within 4 weeks. The descending order of the infection frequency was 98, 96, 92 and 90 as recorded for hypocotyl, leaf, stem explants and the complete seedlings of *H. muticus,* respectively. Furthermore, the growth and quality figure of the obtained hairy root formation were recorded. Hypocotyl explants recorded high value of both root number formation and high figure quality as compared with other of *H. muticus* explants and seedlings. In contrast, as shown in Fig. (1) Strain 15834 infected 80-85 % of exposed explants and seedlings, but caused the formation of kanamycin-resistant tumorigenic calli instead of hairy roots. Although the values for infection frequency and number of hairy roots per explant and quality figure were almost identical for strain A4 hairy roots produced faster than those produced by strain 15834.

Table (1): Effect of different strains of Agrobacterium rhizogenes on the frequency of infection and the growth of Hyoscyamus muticus L. hairy root cultures.

A. rhizogenes strains	Param-eters*	*H. muticus* explants			
		Hypocotyl	Leaf	Stem	Complete seedling
15834 ATCC	1	85±1.73	83±1.24	81±3.25	80±2.97
	2	2.33±0.33	1.25±0.12	---	---
	3	+	+	---	---
A4	1	98±2.35	96±4.95	92±6.45	90±3.21
	2	6.25±1.45	4.15±0.95	1.12±0.15	1.18±0.28
	3	+++	++	+	+

Values represent the mean ± SE of three independent measurements 28 days after inoculation. Approximately 50 explants were examined for each measurement.

*(1)the frequency of infection (%), (2): the number of hairy root formation/explants,(3): the quality figure of the primary initiated hairy roots formation: (+++) high quality, (++) medium quality, (+) low quality and , (-) no response.

Results in Table (1) show that hypocotyl explants are the superior explant tissues for co-cultivation with *A. rhizogenes* strains, because they displayed a greater frequency of infection, and the resulting hairy roots grew more rapidly than those derived from other explants. The strain A4 of *A. rhizogenes* caused infections in almost all exposed explants and seedlings which induced approximately six hairy root initials per explant within 7 days of infection. In contrast to their effect on *Hyoscyamus muticus* L, strain 15834 produced no/or low response of *H. muticus* L. explants or seedlings.

Effect of different concentrations of antibiotic Kanamycin as a selective medium on eliminate of *Agrobacterium* and healthy figure quality

Data presented in Table (2) show that the effect of different concentrations of the antibiotic Kanamycin (mg/l) as a co-cultivation medium on elimination of *Agrobacterium* (strain A4) and healthy cultured hypocotyl explants of *H. muticus*. The results were recorded after 28 days of cultivation. Hundred percent of *Agrobacterium* elimination was recorded with 200 and 250 mg/l, but these concentrations of Kanamycin were unsuitable for obtaining healthy explants. However, the optimum value of the antibiotic (Kanamycin) for both *Agrobacterium* elimination and healthy explants was recorded with 100 mg/l which recorded 97.85 % and 98.5 % for *Agrobacterium* elimination and healthy explants, respectively as compared with other Kanamycin concentrations.

Hypocotyl explants were co-cultivated with A. *rhizogenes* strain A4, for 2 days. Then, explant tissues were transferred to agar-solidified, hormone-free selection medium. Hairy root initials emerged from wound sites on *H .muticus* L explants within 5–7 days after inoculation. After 10–14 days, putative transgenic hairy roots of *H. muticus* L began to grow more rapidly. About 4–5 weeks after co-cultivation with *A. rhizogenes*, hairy roots from hypocotyl henbane explants were excised from the necrotic explant tissues and subcultured on fresh agar-solidified selection medium.

Optimization of culture medium for transgenic hairy root growth

As shown in Table (3), two concentrations (0.5 and 1.0 mg/l) of different auxin analogues were added to the solid MS-culture medium to promote the rapid growth of henbane transgenic hairy root cultures. In general, auxin treatments increased the growth

rate of hairy roots in different patterns. Although rapid growth rates were induced by 1.0 mg l^{-1} IBA or 0.5 or 1.0 mg l^{-1} NAA, these conditions also caused the formation of callus tissues on the transgenic hairy roots. Furthermore, the addition of 1.0 mg l^{-1} IAA, to MS-solid medium containing 3% (w/v) sucrose stimulated the growth rates of root cultures as compared with other auxins. Under these conditions, transgenic roots of hypocotyl henbane explants grew approximately 110 % more rapidly than wild type roots (Fig. 2).

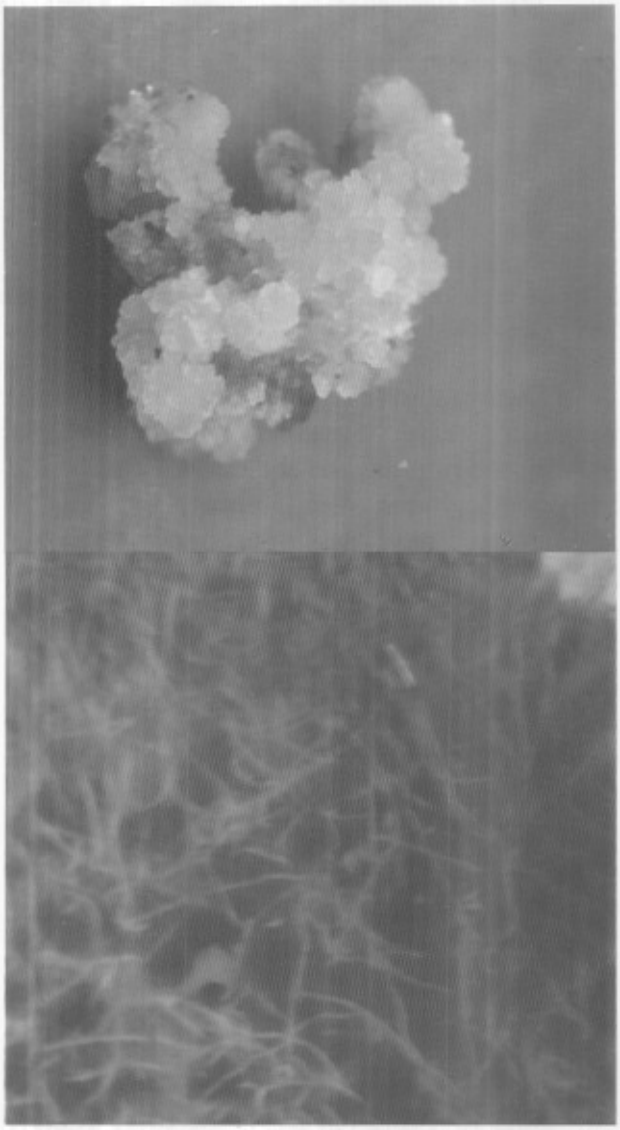

Fig. (1): Tumorigenic calli induced from infected hypocotyl explants of H. muticus with A. rhizogenes strain 15834 .

Fig. (2): Hairy root culture induced from infected hypocotyl explant of H. muticus with A. rhizogenes strain A4 Growth dynamics of the obtained transgenic hairy root culture.

Table (2): Effect of different concentrations of antibiotic Kanamycin (mg/l) as selective medium on elimination of Agrobacterium (strain A4) and quality healthy hypocotyls of H. muticus explants.

Parameters	Antibiotic kanamycin concentrations (mg/l)				
	50	100	150	200	250
% of elimination	75.25±9.24	97.85±2.25	97.65±9.45	100*	100*
% of healthy and quality	96.32±7.75	98.5±2.45	79.6±8.25	---	---

Values represent the mean ± SE and approximately 50 explants were examined for each measurement.
(*)high eliminations which correlate with decline of healthy tissues.

Table (3): Effect of two concentrations (0.5 or 1.0 mg/l) of IAA, IBA and NAA on enhancement of transgenic hairy root growth, derived from hypocotyl explants of H. muticus.

Morphological responses	Auxins (mg/l)					
	IAA		IBA		NAA	
	0.5	1.0	0.5	1.0	0.5	1.0
Callus formation	---	---	++	++	++	++
Hairy root formation	++	+++	+	+	+	+

Where is the percentage of both callus formation and/or hairy root production were: (+) ~ 25-35 %, (++) ~35- 50 %, (+++) ~ 50-85 %.

Data in Fig. (3) Shows that, fresh and dry weight (mg), dry matter content (%) and growth rate (mg/day) of the derived transgenic hairy root cultures from hypocotyl explants of *H. muticus*. These data were used to estimate and determine the different growth dynamics parameters during five weeks of cultivation. The high value 305 and 9.0 (mg) were recorded for fresh and dry weights, respectively after the 5 th week of cultivation. However, the high percentage of dry matter content 3.99 % was recorded after two weeks of cultivation. On other hand, the maximum growth rate 11.71 mg/day was recorded at the end of the 3 rd week of cultivation.

HPLC chemical analysis of total tropane alkaloids, hyoscine and hyoscyamine production in different obtained type cultures

Data in Table (4) show the total tropane alkaloids, hyoscine and hyoscyamine production obtained from callus tissues, tumorigenic calli and transgenic hairy root cultures derived from different explants as compared with control wild plant. These data clearly show that, the highest values of total tropane alkaloids, hyoscine and hyoscyamine (3.25, 0.019 and 0.031 mg/100 mg dry weight) were recorded with tumorigenic calli derived from hypocotyl explants. On other hand, the lowest yield of the previous compounds were recorded with calli tissues derived from the leaf, stem and root explants. The tumorigenic calli derived from hypocotyl explants produced about 1.38 fold of the total tropane alkaloids, 2.37 fold of hyoscine and 2.38 fold of hyoscyamine as compared with wild type plants. However, hairy root cultures derived from hypocotyl explants produced 1.25 fold of total tropane alkaloids, 2.0 fold of hyoscine and 2.15 fold of hyoscyamine as compared with wild type plant of *H. muticus*.

Table (4): HPLC of total tropane alkaloids, hyoscine and hyoscyamine (mg/100 mg dry weight) in calli tissues, tumorigenic calli and hairy root cultures derived from different explants of H. muticus L.

Compounds: (mg/100 mg dry weight)	Calli tissue			Tumorigenic calli			Hairy roots			Wild plant as control
	Leaf	Stem	Root	Hypocotyl	Leaf	Stem	Hypocotyl	Leaf	Stem	
Total tropane alkaloids	1.68	1.09	0.65	3.25	2.84	2.64	2.95	2.54	2.39	2.35
Hyoscine	0.003	0.001	---	0.019	0.011	0.005	0.016	0.010	0.009	0.008
Hyoscyamine	0.005	0.003	0.001	0.031	0.019	0.010	0.28	0.015	0.013	0.013

DISCUSSION

Soil-borne pathogens of the genus *Agrobacterium* are able to transfer part of their DNA (the T-DNA carried on a large plasmid) to the genome of host plant cells. *Agrobacterium rhizogenes* is the causal agent of 'hairy root' diseases in plants, and has been used for the production of hairy root cultures from a multitude of species (Tepfer, 1989). Over the past few decades, transformed root cultures from plants have attracted the attention of many researchers, because of their genetic and biochemical stability, rapid growth rate and ability to synthesize secondary products at levels comparable to wild-type roots. In this work, an efficient *A. rhizogenes*-mediated protocol has been developed for the establishment of transgenic *H. muticus* L. Hairy root cultures. Of two *A. rhizogenes* strains tested, A4 was found to be the most virulent and caused the formation of hairy roots exhibiting the most rapid growth rates(Tables 1, 2).Commonly used strain15834 was highly virulent only on formation of tumorigenic calli rather than roots. Various studies showed also the differential efficiency of various *A. rhizogenes* strains in promoting the formation and hairy roots growth. In agreement with our results, in addition to their variable ability to induce hairy root development, different *A. rhizogenes* strains also affected growth rate, saponin production and the ratio of different astragalosides in transgenic root cultures of *Astragalus mongholicus* Bge (Ionkova *et al.*, 1997). Similarly, Vanhala *et al.*(1995) reported that the strains of *Agrobacterium* also influenced development, growth rate and hyoscyamine production in transformed root cultures of *H. muticus* On other hand, the effectiveness of kanamycin, for the selection of transformed *H. muticus* L. tissue has been investigated by (Belny *et al.*, 1997). Also, in agreement with our results (Sauerwein *et al.*, 1991) reported that the growth rates of transformed roots of henbane was improved by the addition of auxin to the liquid culture medium. Similarly, Loyola-Vargas and Miranda-Ham (1995) found that the exogenous application of auxin was effective to stimulate growth in hairy root cultures of *Lippia dulcis* Trev. In harmony with our results, Nicoll *et al.*, (1995) reported that strain 15834 was the least effective for the induction of hairy roots in *H. muticus*. Transgenic root formation in pea (*Pisum sativum* L.) was dependent on both the strain of *A. rhizogenes* and the genotype of the host plant (Nicoll *et al.*, 1995). Also, Yoshimatsu and Shimomura (1992) and Williams and Ellis (1993) reported that infection of opium poppy hypocotyls with *A. rhizogenes* MAFF 03–01724 and an unidentified strain led to the formation of tumorigenic calli, rather than hairy roots, as observed with strain 15834 . However in contrast with our results Brillanceau *et al.* (1989) reported that strain 15834 was the most virulent and efficient for hairy root development in *Catharanthus roseus* G. Don.

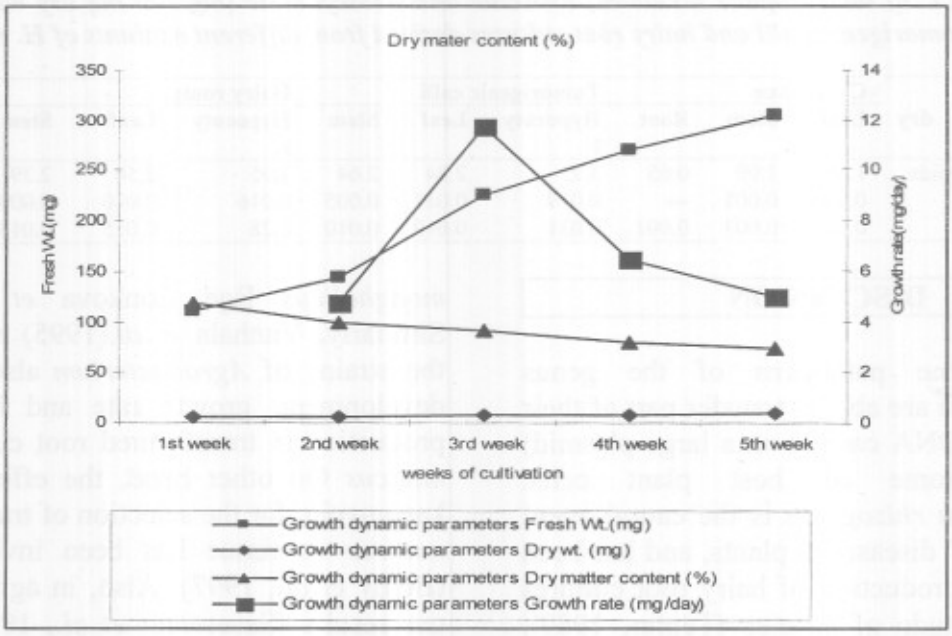

Fig. (3): Growth dynamics of derived hairy root culture from infected hypocotyl explants of H. muticus during five weeks of cultivation. (The initial weight was 100mg).

Clearly, the selection of an effective *Agrobacterium* strain for the production of transformed root cultures is highly dependent on the plant species and must be determined empirically. The differences in virulence, morphology and growth rate are at least partially related to the variety of plasmids contained within each bacterial strain.

Concerning the effect of the transformed hairy root cultures on alkaloids production or other secondary products,in agreement with our results, Yoshikawa and Furuya (1987) reported several transformed root cultures for their content of alkaloids or other secondary metabolites relative to wild-type roots. They reported that hairy roots of ginseng (*Panax ginseng* Meyer) produced the same saponins and ginsenosides as wild-type roots, but in quantities that were 2-fold higher on a dry weight basis). Similarly, Korean balloon flower (*Platycodon grandiflorum* A. DC.) hairy roots produced the polyacetylenes lobetyolin and lobetyolinin at levels 1.60 and 2.6-fold higher, respectively, than those found in wild-type roots (Ahn *et al*., 1996). However, in contrast, with our results, the productivity of the naphthoquinone shikonin in hairy root cultures of *Lithospermum erythrorhizon* (Sieb. et Zucc) was similar to that of wild-type cell cultures, and displayed the same light-dependent control of biosynthesis (Yazaki *et al*., 1998 and Sommer *et al*., 1999). Similarly, *A. belladonna* root cultures have been shown to accumulate hyoscyamine at levels similar to those of wild-type roots (Sharp and Doran, 1990), but also reported the synthesise of the unusual tropane alkaloid littorine which is not found in non-transformed roots (Aoki *et al*., 1997). Moreover, hairy root cultures of *C. roseus* accumulate the monoterpenoid indole alkaloids catharathine and ajmalicine at levels that are also similar to those found in wild-

type roots (Brillanceau *et al*., 1989, Toivonen *et al*., 1989, Vázquez-Flota *et al*., 1994).

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الملخص العربي

تنشيط انتاج قلوانيات التروبان فى السكران المصرى باستخدام اجروبكتريم النقل الوراثى لانتاج الجذور الشعيرية

روفة احمد عبد الرحمن* و حسين سيد طه**

* قسم بحوث المنتجات الحيويه الصيدلانية – مدينة مبارك للابحاث العلمية و التطبيقات التكنولوجية- برج العرب – الاسكندرية- مصر.

**قسم التكنولوجيا الحيوية النباتية- المركز القومى للبجوث- الدقى- القاهرة-مصر.

تم تشييد بروتوكول للنقل الوراثى فى نبات السكران المصرى باستخام الاجروباكتريم لتكوين الجذور الشعيرية، تم اختبار سلالتين هما 15834 و A4 الحاملة للبلازميد pBI 121 لانتاج الجذور الشعيرية من الاجزاء المجروحة من كل من السويقة الجنينية العليا، الورقة و الساق. و تم انتاج مزارع الجذور الشعيرية باستخدام احدى السلالتين بينما السلالة الاخرى انتجت تورما جنينيا و كذلك تم دارسة كفاءة مزارع الجذور الشعيرية المحولة وراثيا فى انتاج قلوانيات التروبان، الهيوسين و الهيوسيامين بالمقارنة بمزاع الكالوس المنتجة سابقا من الاجزاء النباتية المختلفة.