Genetically transformed hairy roots producing agropine induced on *Trigonella foenum-graecum* L. plant by *Agrobacterium rhizogenes* 1601.

انتاج الجذور الشعرية المحَولة وراثيا المنتجة للحامض الاميني الاكروبين (Agropine) في نباتات الحلبة بوساطة *Agrobacterium rhizogenes* 1601

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Abstract

Inoculation of leaves and stems explants excised from field-grown *Trigonella foenum-graecum* L. with engineered *Agrobacterium rhizogenes* 1601 inoculum of optical density 1.90, 2.06 and 1.96 led to the formation of hairy roots on these explants. The highest percent of infection with inoculum of O.D 1.90 was 20% in leaves, and was 53.3% in stems using inoculum of O.D 2.06. Infection percent was 50% in hypocotyl compared with non-inoculated explants. The results showed that inoculation of seedlings lacking roots were slow in hairy root formation and take long time compared with their formation in intact seedlings inoculated with the same inocula. Results of paper electrophoresis of these root proved the incidence of their genetic transformation. Moreover, these transformed roots grow happily in liquid WP medium with an average fresh weight 5.9g after four weeks of culture, whereas fresh weight of normal roots was 1.02g under the same conditions.

المستخلص

أدى تلقيح الأوراق والسيقان المستأصلة من نباتات الحلبة *Trigonella foenum- graecum* L. النامية في الحقل بالبكتريا المعدلة وراثيا *Agrobacterium rhizogenes 1601* بلقاحها ذي الكثافات الضوئية 1.90، 1.96، 2.06 إلى تكوين الجذور الشعرية على هذه الأجزاء النباتية ، وقد سجلت أعلى نسبة للإصابة باستخدام اللقاح ذي الكثافة الضوئية 1.90 وبلغت 20% في الأوراق . وبلغت 53,3 % في قطع السيقان عند تلقيحها باللقاح ذي الكثافة الضوئية 2,06 مقارنةً بالكثافات الأخرى . وسجلت نسبة 50% في السيقان تحت الفلقية بالمقارنة مع نظيراتها غير الملقحة . وقد بينت النتائج إن تلقيح بادرات الحلبة الفاقدة لمجاميعها الجذرية بطأ تكوينها للجذور الشعرية واستغرقت وقتاً أطول مقارنة بتكوينها في البادرات الكاملة . وأكدت نتائج الترحيل الكهربائي الورقي لهذه الجذور تحولها الوراثي بدلالة الكشف الايجابي للاكروبين . واتصفت هذه الجذور بنموها الغزير في وسط WP السائل بنصف قوته التركيبية وسجلت معدلات أوزانها 5.97 غم في الأسبوع الرابع ، بينما كان معدل الوزن الطري 1.02 غم للجذور الاعتيادية في نفس الظروف .

Introduction

Agrobacterium rhizogenes is a Gram-negative soil bacterium, responsible for adventitious (hairy) roots formation upon agroinfection and accumulation of biochemicals in plant metabolism [1]. Root induction is due to stable integration of the Ri-tDNA (transferral DNA) into the plant genome and its subsequent expression. Transformation process produces available by-product hairy roots, which formed at or

near the site of infection, in addition, opines are produced and serve as specific food for the bacteria [2]. *A.rhizogenes*-derived hairy roots have numerous applications in many areas of research. For example, cultures of hairy roots have been used extensively in root nodule research [3], production of industrial products [4], and as experimental systems to study biochemical pathways [5]. Hairy root cultures have three main characters: genetic stability, cultivation in free growth regulators media and ability to give high biomasses [6].

Trigonella foenum-graecum L. (fenugreek, Hulabab) is a forage legume, used as a green manure and source of medicinally important steroid sapogenins [7] such as diosgenin, yomogenin, gitogenin, tigogenin and neotigogens. It is a good source of bioactive constituents such as mucilage, volatile oils, choline and trigonelline [8].

This study aimed to produce transformed hairy roots cultures via agropine type *Agrobacterium rhizogenes.* These cultures will used to collect specific secondary metabolites which will publish in the next article.

Materials and Methods

- **Plant material.**

Trigonella foenum-graecum L. seeds were obtained from local market. Seeds and explants excised from field-grown plants were surface sterilized with 3% sodium hypochlorite NaOCl solution for 5 min. and rinsed three times with sterile distilled water. Sterilized seeds were germinated on agar-solidified MSO medium [9] and maintained in dark for three days at 25^{o}C. After seeds germination, plantlets were transferred to agar-solidified half-strength McCown's woody plant (WP) medium [10] they maintained at 25^{o}C in the light at 700 lux with a daily 16h photoperiod [11].

- **Bacterial strain and inoculum preparation.**

Agrobacterium rhizogenes R1601 (supplied by Professor. E.W. Nester, Washington univ., USA) harbouring Ri plasmid and carrying Kanamycin and Carbencilline resistance genes. Single colony was transferred to 25 ml of APM liquid medium [12] and incubated at 25^{o}C for 24, 48 and 72 h in the dark on a rotary shaker at 120 r.p.m. Bacterial suspensions were centrifuged (1200 r.p.m. for 15 min); pellets were resuspend in 1.0 ml freshly prepared APM liquid medium. The produced inoculate have the optical densities (O.D) 1.90, 2.06 and 1.96 were used for inoculation of explants.

- **Inoculation of explants and induction of hairy root culture.**

Axenic explants were inoculated by direct injection [13] using a fine needle (0.25×9.5mm) immersed in the inoculum and pricked each sample. Leave explants were inoculated in the midrib whereas stem explants and hypocotyl were injected at the tops. Inoculated samples were cultured vertically in agar solidified half-strength WP medium and maintained at the same conditions mentioned previously[13]. Hairy roots formed on inoculated samples were excised and transferred to agar solidified half-strength hormone free WP medium supplemented with gradual concentrations 100, 200,300, 400 and 500 mg / l of Cefotaxine [13] to eliminate bacteria.

Small clusters of hairy roots were transferred to 50 ml 1/2 strength hormone-free WP liquid medium and incubated on rotary shaker at 80 r.p.m. in dark at 25° C to assess the growth rates.

- **Paper electrophoresis for Agropine detection.**

Standard method [14] was used in the detection of opines. Samples of 100 mg of hairy roots, and of normal roots, were separately crushed in the presence of 0.1ml 0.1N HCL in Eppendorrf tube, centrifuged for 20 min. at 6000r.p.m. Ten micro liter of supernatant of each samples and 2 µl of standard agropine (CNRS France) were spotted on paper (Whatman No.3), and laid in the basin of electrophoresis tank using buffer solution consisting of formic acid: acetic acid: water (5:15:80 ml). Voltage of (300-400)v for 1h was pass-through. The chromatogram was removed, dried in the air, stained with silver nitrate($AgNO_3$) solution, dry again for 30 min., then submerged in a solution of 2% Methanolic NaOH to develop spots, dry and soaked in solution of 5% $Na_2S_2O_3$, washed with running water for 30 min., then dried by air

Results

- **Development of hairy root cultures.**

Generally results expressed that there is a consensus between *T. foenum-graecum* and *A. rhizogenes.* Stem explants excised from field-growing plants give a clear response to agrobacterial inoculation compared with the response of leave explants Table (1).

Table (1): Response of leaves and stems explants excised from field grown *Trigonella foenum-graecum* plants to inoculation with *Agrobacterium rhizogenes* 1601 of different O.D.

Explants	O.D	No. of infected explants	Response (%)
Stems	1.90	1	6.60
	1.96	6	40.0
	2.06	8	53.3
Leaves	1.90	3	20.0
	1.96	0	0.0
	2.06	2	13.30

Each treatment had 15 replicates. , O.D: optical density.

The results indicate that hairy roots were formed on inoculated and non inoculated sites of stems Figure (1.A) and leaves Figure (1.B) explants. The removal of these hairy roots and subculture them on agar solidified WP medium gave an efficient culture of white color hairy roots and they were negatively-geotropism in their growth Fig (1.C and D).The main observation was the difficulties in removing bacteria (of optical density 2.06 and 1.96) from root culture, therefore inoculum of O.D 1.90 was used in the next experimentation

Data indicate that inoculation of hypocotyl explants with *A. rhizogenes* of the same O.D sustained the formation of hairy roots in different locations with an average number of 2.1 root/ explants compared with control samples inoculated with distilled water only that remained alive with no root formation Table (2).

Fig (1): Formation of hairy roots on explants of *Trigonella foenum- graecum* L. inoculated with *A. rhizogenes.* (A)Induction of hairy roots on stem explants grown in WP medium. (B)Induction of hairy roots on leaf explants grown in WP medium. (C) Culture of stem hairy roots induced in (A) grown in WP medium (4 wks old). (D): Culture of leaf hairy roots induced in (B) grown in WP medium (4 wks old)

Table (2): Formation of hairy roots on hypocotyls explants excised from *Trigonella foenum-graecum* seedlings to inoculation with *Agrobacterium rhizogenes* 1601.

Explants	No. explants			Exit points of hairy root		Average No. H.R.
	Inoculated	Infected	Non-infected	Inoculated sites	Non- inoculated sites	
Hypocotyls	20	10	10	7	3	2.1
Control	10	0	10	0	0	0

Control sample were inoculated with water.

The results showed that inoculation of seedlings lacking root systems with Agrobacterium inoculum of O.D 1.90 encourage the formation of hairy roots within 11 days. Whereas it takes 14 days to develop on intact seedlings inoculated with the same inoculum Table (3).

Table (3): Response of *Trigonella foenum-graecum* seedlings to inoculation by direct injection method with *Agrobacterium rhizogenes* of O.D 1.90.

Sample	No. seedlings			No. of Explants produce H.R.	Time required (day)	Average No. H.R.
	Inoculated	Infected	Non-infected			
Intact seedlings	20	11	9	7	14	2.57
Seedlings lacking roots	35	20	15	18	11	4.67
Control	20	0	20	--	--	--

Control samples of both types of seedlings were inoculated with water.

The developed hairy roots on intact seedlings Figure (2.A) and on root decapitated seedlings Figure (2.B) were white in color with dense of root hairy Fig (2.C).Transfer of small segments of hairy roots to the surface of agar-solidified WP medium produced an active culture Figure (2.D).The results showed that transfer of this

culture to WP liquid medium encourage their growth Figure (2.E) whereas normal roots exhibit limited growth in the same medium Figure (2.F)

Interestingly, a clear increasing in fresh weight of these hairy root grew in 1/2 strength liquid WP medium after four weeks of culture Table (4).

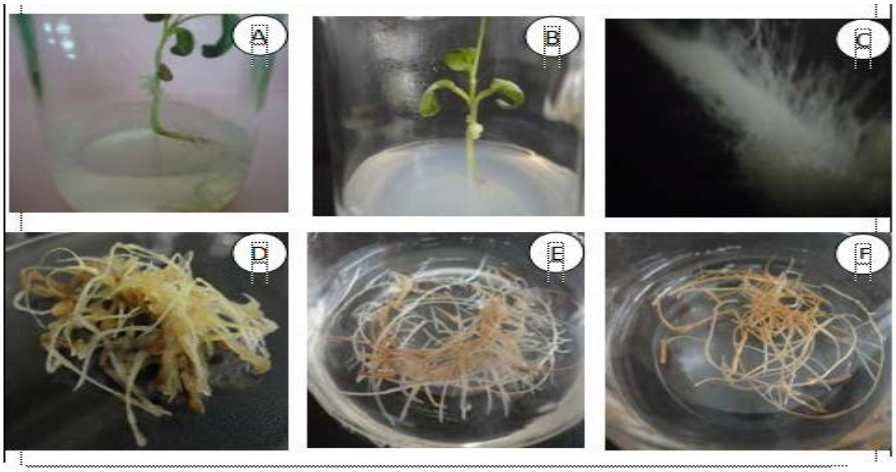

Fig (2): Production of hairy roots on seedlings of *Trigonella foenum-graecum* L. inoculated with *A. rhizogenes* and their growth in liquid culture. (A) Stimulation of hairy roots on Intact seedlings grown in WP solid medium. (B) Stimulation of hairy roots on seedlings lacking roots grown in WP solid medium. (C) Single hairy root in (A), note the dense of root hairs.(D) Culture of hairy roots produced from roots in (B) grown in WP solid medium. (E) Efficient growth of 4wks old hairy roots in WP liqiud medium. (F) limited growth of 4 wks old normal root in WP liquid medium.

Table (4): Biomass fresh weight rate of agrobacterium genetically modified hairy roots and normal roots of *Trigonella foenum-graecum* in 1/2 strength WP liquid medium.

Samples	Average biomass fresh weight(g)			
	Initiation	2 weeks	3weeks	4weeks
Hairy roots	0,31	0.56	1.86	5,97
Normal roots	0,32	0.49	0.72	1,02

- **Detection of Agropine in hairy roots.**

The chromatogram results showed the separation of black spots from the extraction hairy roots samples. They developed corresponding to the spots formed by standard agropine with failure of spot separation from normal root samples Figure (3).

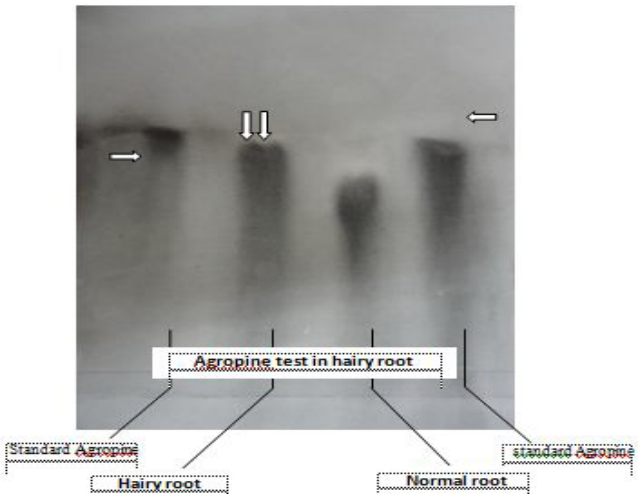

Fig (3): Chromatogram of agropine detection in hairy roots produced on *Trigonella foenum-graecum* L. by *Agrobacterium rhizogenes1601* by paper electrophoresis using $AgNO_3$ solution.

- **Characterization of hairy root.**

Transformed hairy roots formed on leaves and stems explants were white in color, with numerous branches and having dense of root hairs. Additionally they were negatively –geotropism in their growth. On agar-solidified WP medium Figure (2,C,D).

Discussion

The appearance of hairy roots at inoculation sites with *Agrobacterium rhizogenes* R1601 on explants and seedlings attributed to the transition of a segment of T-DNA of Ri plasmid to the plant cell [15] and its integration with the host genetic material in the form of single copy and multiple copies similar to the division of plant cell where it is present [16], and to the genes expression to exhibit the first signs of genetic transformation of plant cell by development of transformed hairy roots [17].

Response of both intact seedlings and those lacking roots to infection by direct injection with *A. rhizogenes* R1601 may due to the different of endogenous content of growth regulators in these seedlings and may also due to the architecture of genes and to the large number of parenchyma cells in leaves compared with their number in stems explants [18].The required time for incidence of hairy roots varies from one to several weeks depending on the plant species factor, and on the bacterial strain and their interaction [19]. This speculation explained the development of transformed hairy roots on various plant species involving different time, for example on *Trigonella foenum-graecum* they emergence after 19 days of inoculation [20], and after 21 days in *Beta vulgaris* [21] using the strain 1601 of *A. rhizogenes.*

In this study the presence of agropin in hairy roots confirms the genetic transformation of these tissues [22]. The synthesis of this unusual amino acid occur as a result of the transition of T-DNA segment of Ri plasmid containing the genes controlled the agropin synthesis [23].The rapid growth of these transformed hairy roots and its dense of root hairs, may explained to the length of apical meristem of these roots [19], as well as to the increased rates of cell division [24].

The conclusion is transformed hairy roots represent an efficient alternative approach to regenerate into plants, often spontaneously, such as occur in *Brassica oleracea* [25] and in *Solanum nigrum* [26]. Also, in the production of plant secondary metabolites in cell cultures instead of organized or differentiated cultures with evidence that genetically transformed roots can produce numerous secondary metabolites [27] compared with the whole plant. Furthermore, they grow faster than the untransformed roots or cell cultures and they show a long-term stable production of these medicinal plant products [28].

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