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A Protocol for the production of chickpea plant resistant to *Fusarium* wilt from genetically transformed hairy roots

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Abstract:

Data demonstrated that the relation between root-decapitated chickpea seedlings and Agrobacterium rhizogenes R1601 harboring Ri-plasmid was successful. This is expressed through the formation of shiny clusters of white and negatively geotropism hairy roots. These adventitious roots were excised and cultured on solid MSO medium. Hairy roots extract was electrophorized on chromatographic paper proved the separation of agropine that represent a strong marker of transgenesis of hairy root tissues. Callus production from these tissues in the absence of growth regulators is an additional evidence of callus transformation. This callus was difficult to regenerate, and the produced chickpea plants were morphologically abnormal. They need to be tested whether they were resistant or tolerant the infection with Fusarium and / or Aschochyta.

Key words: Agrobacterium rhizogenes 1601, Agropine

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Introduction

It is well-known that utilization of callus tissues in the production of plant led to various soma-colonal variations (Larkin and Scowcroft, 1981). Such plants might be resistant to different pathogenic fungi (Jin,*et al*., 1996). Recently many studies successes in developing new plants through transferring single or cluster of gene(s) to the plant genome(Glick and Pasternak, 2003). The genetically transformed grain-legumes considered of important agronomical and economical traits. This is aimed to improve plant species such as the transfer of biotic stress GST and Letpx1 genes via *Agrobacterium tumefaciens* vector in mature embryos and production of transgenic chickpea plants (Ibrahim, 2006). *Agrobacterium rhizogenes* mediated-transformation of legumes aimed to transfer interested genes acquiring these plants resistance to fungal diseases such as in pea (Lawrence and Koundal, 2000). Most studies indicated that callus was spontaneously produced from genetically transformed hairy roots .Subsequently, this callus was stimulated to produce *Lotus corniculatus* new plants a reported with (Petit *et al.,* 1987), *Trigonella* (Al-Mahdawi, *et, al*., 2013).

The need for this study is to produce chickpea plants from transformed hairy roots derived –callus.

Materials and Methods

Plant materials

Seeds of chickpea, Shawki cultivar were obtained from local company. They were surface sterilized with NaOCl (Mohammed *et.al.* 2011). Seedlings were produced on agar-solidified MS (Murashige and Skoog, 1962) medium in culture room at 25±2 °C, 16 h illumination conditions.

Preparation of *A.rhizogenes* R1601 Inoculum

This strain harboring Ri-plasmid containing Kana. $^{(Res+)}$ and Carb $^{(Res+)}$ genetic markers-(supplied by Prof. E. W. Nester, Univ. of Washington. USA).This bacteria was kept on agar-solidified APM (Morgon, *et al.*, 1987) medium containing 100mgL-1of each Kanamycin and Carbencillin Bacterial inoculum was prepared by transferring a loopful of bacterial growth to 100 ml of liquid APM medium provided with the same types and conc. Of antibiotics. Flasks were incubated on shaking incubator (New Brunswick Scientific Co. Inc., Edison, USA) at 28° C for 48 h, 150 rpm. Bacteria were harvested by centrifugation. To the bacterial pellet fresh APM medium was added .Optical density of this suspension was measured at 600 nm (Al-Mallah and Masyab, 2014).

Seedlings direct injection

Thirty – Day's old root-decapitated chickpea seedlings were each inoculated by injection into 3-4 positions by sterile syringe needle dipped in *A. rhizogenes* inoculum. These inoculated seedling were cultured vertically in agar-solidified MSO medium (Al-Mallah and Mahammod, 2012) in glass jars of 100 ml volume, Specimens were kept in culture room (22+ C, 1000 Lux diffused illumination for 16 h.).

Bacterial Free-hairy roots formation

Two-three cm length hairy roots that developed at inoculation sites were aseptically excised. Each root was cultured on the surface of 20 ml of agar-solidified MSO medium in 9.0 cm diam. plastic Petri-dish (Sterilin, UK). In these cultures when bacterial growth was noted hairy roots were recultured soon on the same medium supplemented with gradual conc. 250, 300, 350 and 400 mgL-1 of cefotaxime. They kept on each conc.15 days. When hairy roots became free-from bacteria they were sub cultured again on agar solidified MSO medium. To check that hairy roots became free from bacteria 0.5 mg of roots was

minced in 1.0ml of liquid APM medium in eppendorff tube. 50 ul of this preparation was transferred to liquid APM medium; samples were incubated in dark (David, *et.al.,* 1984).

Determination of agropine by paper electrophoresis

The unusual amino acid "agropine" was detected in these hairy roots and callus by paper electrophoresis (Tefer and Tempe, 1981). Electrophoresis conditions were 300-400 volt for one hour. The buffer solution was consisted from 60 ml acetic acid: 30 ml Formic acid: 910 ml distilled water (Al-Mallah and Mohammed, 2012; Al-Mallah, *et.al.* 2013). Electrophortogram was washed, dried, immersed in silver nitrate solution, washed and dried.

Results

Conservation of Genetic Markers:

The results indicate the presence of kana.$^{(Res+)}$ and carb.$^{(Res+)}$, genes on Ri-plasmid. This was expressed through the capability of *A.rhizogenes* R1601 to grow on APM medium provided with 100 mgL-1 of each antibiotic.

Establishment of adventitious hairy roots:

The main observation that the injected seedling remains viable. Their response was the stimulation of adventitious hairy roots from the inoculated and uninoculated sites .The formation of these roots was affected by bacterial suspension density (Table, 1).

Table(1):Formation of hairy roots on root-decapitated chickpea seedlings (*Cicer arietium* L.) by direct injection with *Agrobacterium. rhizogenes* R1601.

Bacterial Density (x10³cell/ml)	Total no. seedlings Inoculated / Infected		Root Formation (%)	Root Number	Time (day)
0.0	50	0.0	0.0	0.0	0.0
365	76	7.0	92	2-6	28
275	72	24	33	6	10
96	88	36	40	6-8	12
90	60	15	25	4	21

These data pointed out the role of agrobacterial infection in the stimulation of hairy .They appeared as white thread –like structures and were negative geotropism in their growth. Then they developed to cluster of tiny and hairy roots upon inoculated seedling (Fig.1.A) .It was noted that the transfer of individual hairy roots of 2.3 cm length to solid MSO medium they grow happily and increased in length (Fig.1.B).Sequential transfer of these roots on gradual conc. of cefotaxime produced bacterial –fee hairy root culture, Moreover, growth of hairy roots on auxine-free MS medium refer that they are transgenic coupled with the beginning of callus-like structure formation (Fig.1.C).

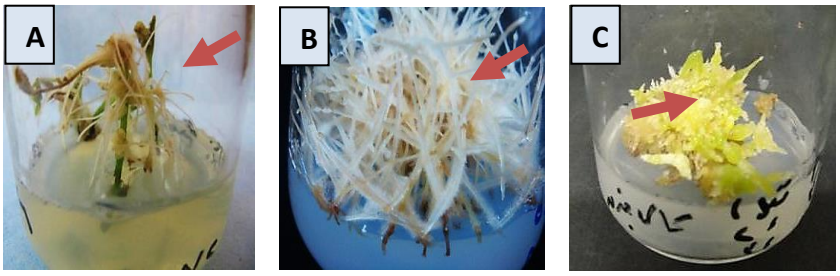

Fig.(1) Formation of hairy roots on chickpea seedlings(Local Shawki cult.) by *Agrobacterium rhizogenes* R1601.

A-Induction of hairy roots (arrowed) at inoculated sites of seedlings grown on MS medium.

B-Culture of hairy roots in (A) grew on MSO medium.

C-Callus-like structures (arrowed) spontaneously formed from hairy roots.

Agropine isolation:

Electrophoretogram exhibited the separation of black spots corresponding to the spots of standard agropine (Fig.2). Development of such spots from hairy root and callus –like structures represent an additional evidence that those tissues were genetically transformed .Samples of both normal roots and callus failed to exhibit similar spots (Fig.2) .

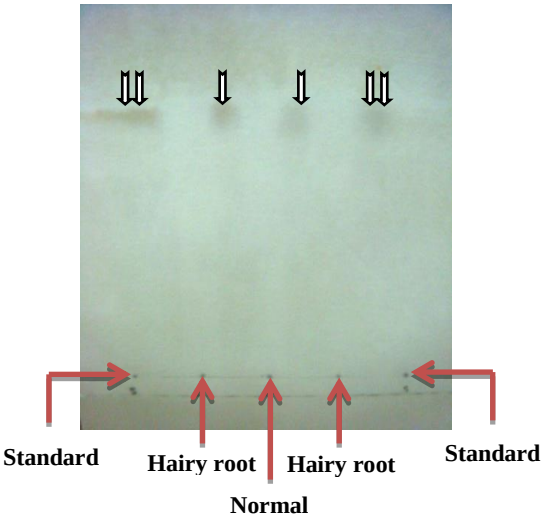

Fig. (2): Isolation of agropine from transformed hairy roots and its callus by paper electrophoresis.

Spontaneous formation of calli:

The results indicate the spontaneous formation of callus from transformed hairy roots without the need for auxin addition. This transformed calli was yellow in color and rapidly propagated on MS medium supplemented with 4.0mgL^{-1} TDZ. This medium stimulates the development of few and small organs –like structures (Fig.3.A).

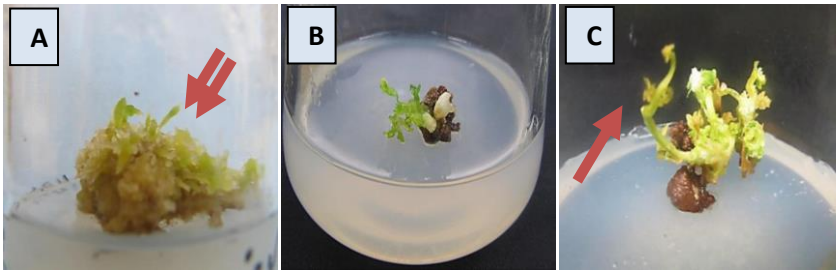

Fig. (3): Differentiation of transformed hairy-root derived-callus of chickpea
A-Formation of shoot-like structure (arrowed) from hairy root callus on regeneration medium.
B-Development of shoots in (A) on MB+ 2.0mg-l-1 BA+3.0mgL-1GA3.
C-Growth of shoots in (B) Note the abnormal leaves (arrowed).

Regeneration of chickpea plant from transformed hairy roots.

The results referred to the difficulty of agropine –positive callus differentiation. Long –term subculture of this callus on the regeneration medium MB+2.0mgL^{-1} +3.0mgL^{-1} GA_3 support the proliferation of single shoots(Fig.3.B).They were abnormal in their growth and held distorted and thicked epidermis leaves (Fig.3.C).

Discussion

The role of *A.rhizogenes* R1601 in producing putative transformed chickpea plants from agropine-positive hairy roots certainly due to the host-bacteria compatibility (Senthil, *et.al*.2004). This is explained to the transfer of bacterial T-DNA genes responsible for the hairy root stimulation .Integration of these genes with genomic DNA of plant cells may led in the genetic transformation (David *et al*, 1984). Other genes of Ri-plasmid named *Vir*-genes controlled the infection and T-DNA integration in nuclear material of plant (Hooykas and Schilperoot,1992). The separation of the unusual amino acid "agropine" from these root is due to the opines synthesis genes which transferred from T-DNA to the transformed plant cell

(Savka and Farrand, 1992; Al-Mallah and Masyab, 2014). Additionally hairy roots formation involved the active transport of *iaaM* and *iaaH* genes , also *tmr* genes. This cluster of genes are localized on Ri-plasmid are sustained the formation of hairy roots (Meyer, *et al*. 2000). Spontaneous calli formation from these roots in the absence of growth regulators in nutrition medium represent a selection culture to distinguish between the transformed and normal calli (Ibrahim, 2006). In this study, the production of morphologically abnormal plants probably due to the active expression of *rol A* genes in stem cells and its weak expression in leaf and root callus (Ignacimuth and Prakash, 2006). Other investigators explained this phenomenon to the low level of cytokinnes and high level of gibberellins in these transformed plants compared with non-transformed plants. Generally, the regeneration difficulties in chickpea plant species is likely joined with the endogenous hormones produced by transformed tissues in culture. The later may prevented or act as a barrier of callus differentiation (Veluthambi, *et.al*.2003). The conclusion of this study that chickpea plants regenerated from transformed callus are expected to be resistant to wilt-causing fungi.

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