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Establishment of rapid propagation cultures of *Agrobacterium rhizogenes* – mediated transgenic hairy roots in sugarbeet

AL. NEMA Q.S.[1]
AL. MALLAH M.K.
Biotechnology Laboratory
College of Education for Pure Sciences
University of Mosul, Iraq

Abstract:

The clones (C1, C2, C3 and C4) of sugarbeet leaves express their capability to induce the adventitious roots as a result of their injection with suspension of Agrobacterium rhizogenes R1601. Clear differences were found in the response and transgenesis of these explants. The result indicated that C3 was the best in this case, but hairy roots produced from all clones have similar phenotype. Generally, numerous cultures of agropine – positive and rapid propagation hairy roots were obtained.

Key words: *Agrobacterium rhizogenes*, hairy roots, sugarbeet

INTRODUCTION

Agrobacterium rhizogenes is an efficient experimental vector in producing transgenic plant tissue (Hooykass and Schilperoort, 1992). This bacteria can donate and transfer foreign DNA into plant cell based on the autonomous root – inducing (Ri) plasmid

[1] Unsheathed from Ph. D. Thesis.

and produce hairy roots (AL-Mallah and Mohammed, 2012). Recent study performed in tomato and potato proved strictly the transfer of T-DNA from this bacteria to both tyres using reporter (*GUS* and *GFP*) genes technology (AL-Mallah and Masyab, 2014). *Agrobacterium rhizogenes* mediated gene transfer is widely used to obtain hairy roots in many plant species as in carrot (AL-Mallah and Mohammed, 2012), Fenugreek (AL-Mahdawe *et al.*, 2013) and cowpea (Rasheed and Abdullah, 2013). Different phenotypes of hairy root were produced, and some of them with high production of useful metabolite (Ali *et al.*, 2012). Generally, sugarbeet (*Beta vulgaris* L.) is one of the important industrial crop, and the need for this investigation was to create transgenic hairy roots from leaf clones of this plant species and to select a rapid propagation cultures.

MATERIALS AND METHODS

Agrobacterium rhizogenes R1601

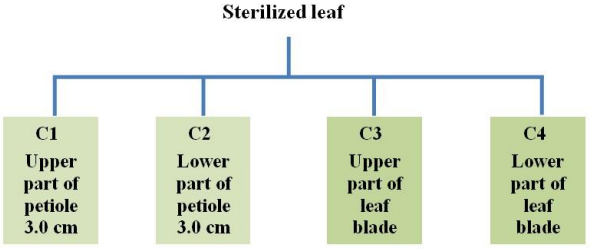
This strain that harboring Ri-plasmid with Kana.$^{Res+}$ and Carb.$^{Res+}$ genetic markers available in Plant Genetic Manipulation Lab. was supplied from Professor E. W. Nester (Washington University, USA). This bacteria was grown on agar-solidified APM medium provided with 100 mgl^{-1} of each antibiotics (Morgan *et al.*, 1987).

Preparation of leaf clones

Fully – expanded leaves were excised from 12 weeks old field – grown sugarbeet plants of the Swedish variety Baraka (obtained from the General Enterprise of Sugar, Mosul, IRAQ). The separated leaves were soaked in 200 ml of 70% ethyl alcohol for two min., then immersed directly in 200 ml of 1.5% NaOCl for five min., and they washed thoroughly with autoclaved water (Ritchie *et al.*, 1989).

Direct injection of explants with bacteria

Agrobacterium rhizogenes R1601 suspension was prepared as described (AL-Nema, 2001). A group of sterilized leaves similar in sizes were selected. Each leaf was cut into four parts as in diagram below:



Each explant was inoculated by injection with needle (0.25 × 9.5 mm in size) already its end was immersed in *Agrobacterium* suspension. Petioles explants were inoculated at their tips whereas leaf explants were inoculated in midribs in similar method of AL-Mallah and AL-Nema (2001). Control samples were inoculated with distilled water using the same manner.

Establishment of bacterial – free hairy root culture

Clusters of hairy roots 1.0 – 1.5 cm in length were cut from stimulation sites and placed on 20 ml of agar solidified MSO (Murashige and Skoog, 1962) medium in glass jars. As bacterial growth was noted in hairy roots cultures they were each transferred to plastic Petri-dishes (9.0 cm diam.) containing 20 ml solid MS medium provided with 50, 100, 150, 200 mgl^{-1} cefotaxime. They stay on each concentration 15 days until the cultures became free from bacteria (AL-Mallah and AL-Nema, 2012).

Transgenesis of hairy roots

A. Conservation of genetic marker on Ri

This test was carried out routinely every three months following discs technique. Antibiotics saturated paper discs

were spread on the surface of agar solidified APM medium previously streaked with 0.1 ml of bacterial suspension (Bauer *et al*., 1966).

B. Detection of opines in hairy roots

Hairy roots sample of 100 mg weight was homogenized in the presence of 100 µl of 0.1 N HCl. Then the supernant was electrophorized on chromatographic paper (Electrophoresis, Esselte Studium S-11285 Stockholm, Sweden). Electrophoretogram was removed, dried and treated with the developing solution to detect the agropine spots (Tepfer and Tempe, 1981).

RESULTS

Conservation of *A. rhizogenes* their genetic markers

The results referred to the growth of these bacteria when cultured on agar – solidified APM medium provided with 100 mgl^{-1} of each kana. and carb. This demonstrates that bacteria still harboring Ri-plasmid that conserved resistance genes of these genetic markers.

Hairy roots cultures induction

The primary observations proved the success of leaf explant inoculation that injected with *A. rhizogenes* R1601 suspension. Moreover, these tissues remained viable and tolerated bacterial infection. Development of hairy roots began as adventitious roots on inoculation sites of the various clones that exhibit different response (Table, 1).

Table (1): Comparison of hairy roots development on the different clones of sugarbeet leaves directly injected with *A. rhizogenes* R1601

Explants		*No. of explant forming root	Stimulation (%)	Mean number Root / explant
Petiole	Clone 1	42	84	5.0
	Clone 2	40	80	9.0
	(Control)	0.0	0.0	0.0
Blade	Clone 3	26	52	12
	Clone 4	23	46	3.0
	(Control)	0.0	0.0	0.0

*No. of explant 50 / treatment

Visual examinations indicated the induction of hairy roots as short thread – like structures and possess dense of root hairs on Clone 1 that represent the upper portion of leaf petiole (Fig. 1, A). Also, similar hairy roots were developed on the inoculated and un-inoculated sites of Clone 2 (Fig. 1, B). Again, hairy roots formed on leaf blade were similar to those formed on petiole explants but they were longer (Fig. 1, C) and less in their number on Clone 4 (Fig. 1, D). Generally, petiole explants was in the first order in their response to bacterial infection.

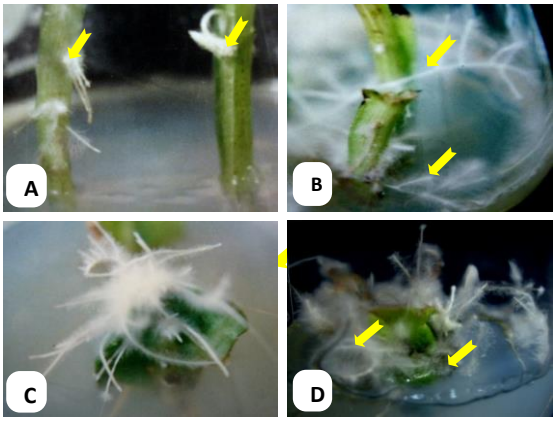


Fig.(1) Stimulation of hairy roots on various clones of sugarbeet leaves grown on solid MSO medium inoculated by direct injection with *A. rhizogenes* R1601.

A) Induction of hairy roots (arrowed) on C1 of leaf petiole.

B) Hairy roots stimulated (arrowed) from un-inoculated positio of C2.

C) Formation of hairy roots on C3 of leaf blade after 15 days of inoculation.
D) Development of hairy roots (arrowed) on inoculated and un-inoculated sites of C4 explants.

Production of bacterial – free hairy root culture

The results proved that excision of 15-20 days old clusters of hairy root, and then each one transferred on the surface of agar - solidified MSO media provided with the gradual conc. of 50, 100, 150 and 200 mgl^{-1} of cefotaxime eliminate the bacteria and produced cured cultures of hairy roots.

Detection of agropine in transformed hairy roots

Data of paper electrophoresis demonstrated the separation of dark black spots of agropine (unusual amino acid) facing the spots of standard agropine. Differences was present in the separation of this opine from hairy roots tissues of the various clones (Table, 2). These results confirmed the transgenesis of these hairy roots cultures.

Table (2): Differences in agropine spots separated by electrophoresis from hairy roots developed on sugarbeet clones inoculated by *A. rhizogenes*

Source of hairy root	Number of electrophoresis	Diameter of spot (cm)	*Reaction
Clone 1	**2**	**0.5**	**(+)**
Clone 2	**3**	**0.5**	**(+)**
Clone 3	***3***	**2.0**	**(+++)**
Clone 4	***2***	**2.0**	**(++)**

*** (+++): very clear, (++): clear, (+): weak**

Hairy roots phenotype

Cultures of hairy roots were white in color, rich with root hairs, having rapid growth with numerous branches and were negatively geotropism (Fig. 2). Moreover, they dried easily when exposed to air which stand as a problem.

Fig.(2) Production of agropine – positive rapid propagation transgenic hairy root cultures (2 weeks old) of sugarbeet derived from C3 explants directly injected with *A. rhizogenes* R1601.

DISCUSSION

The reason behind the production of rapid propagation transgenic hairy root cultures may explained to the efficiency of bacterial strain used in this work (AL-Harbawy and AL-Mallah, 2015). Also explant type strongly affected the success of infection method (AL-Mallah and Mohammed, 2012), and mainly to the Ri plasmid copy number harbored in the strain R1601 of *A. rhizogenes* (AL-Mallah and Masyab, 2014).

In this investigation the similar treated clones exhibit different response, this may due to the *rol* – genes (*rol* A, *rol* B, *rol* C and *rol* D) of Ri plasmid which controlled the induction of these hairy roots (Young *et al*., 2012). These genes and T-DNA orientated the synthesis of substances stimulated the infected plant cells to differentiated into root structure uder the influence of endogenous auxins (Ooms *et al*., 1986). This may be coupled with the effect of Ri T-DNA containing *tra* loci that directly synthesize auxin (Capone *et al*., 1989) and induce hairy

root formation. Generally, petiole clones were more amenable to agrobacterial infection since they offer insuranced sites for bacterial penetration emphasising the fact that wound response was an essential requirement for transformation event (Potrykus, 1990).

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