IJST EXPLORE International Journal of Science and Technology
CENTRE OF PROFESSIONAL RESEARCH PUBLICATIONS
ISSN 2049-7318

International Journal of Science and Technology Volume 4 No. 6, June, 2015

Transformation of Soybean (Glycine Max L.) Via GUS –Labeled Agrobacterium Rhizogenes R1000

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ABSTRACT

The objective of this work was to investigate the ability of *GUS* –Labeled *Agrobacterium rhizogenes*-strain R1000 for transformation to produce hairy roots in *Glycine max* L. .Two different methods were tested to perform the infection co-cultivation and direct inoculation . Cotyledons ,hypocotyls , epicotyls and leaves from seedlings of soybean were used as explants. In this study the direct inoculation was the best method .The cotyledons were showed a higher percentage for the formation of hairy roots, which were 76.9%, followed by hypocotyls , epicotyls and leaves which showed percentages 64.0% ,58.3% and 38.0% respectively .Histochemical assay proved that *GUS*- gene was transferred to hairy root cells .

Key words: *Soybean; Transformation; Agrobacterium Rhizogenes; Strain R1000; Hairy Roots.*

1. INTRODUCTION

Soybean [*Glycine max* (L.) Merrill], one of the world's most important crops due to the high content (40%) of protein and about (20%) oil in its seeds, is now the first legume species with a complete genome sequence (Schmutz *et al.*, 2010). The first successful transformation of soybean was reported (Hinchee *et al*., 1988), two major methods have been used in soybean transformation: one is particle bombardment of embryogenic tissue and the other is *Agrobacterium tumerfaciens*-mediated transformation. In recent years, a new transformation method has been developed which represented a significant advancement due to its less time consuming to generate transgenic plant tissue . Hairy root transformation offers the advantage over *A.tumefaciens*-mediated transformation every transgenic root represents an independent transformation event, high numbers of transformants can be obtained and analyzed in a relatively short period of time.

Agrobacterium rhizogenes causes hairy-root disease of plants in a manner similar to the crown-gall disease caused by *A.tumefaciens*. Both infect at wound sites and transfer T-DNA from the bacterial cell to the plant cell. Integration and expression of this DNA in the plant genome leads to the development of the hairy-root phenotype (Grant *et al*., 1991) and synthesis of novel low-molecular-weight compounds called opines (Petit *et al*., 1983). Hairy-root cultures grow rapidly, show pleiotropic root growth and are highly branched on hormone-free medium.

In recent years, soybean has been transformed by using different explants (Wang and Xu, 2008 ; Liu *et al*., 2013) and various factors affecting the transformation efficiency have been optimized (Xue *et al*., 2006; Liu *et al*., 2008).

The objective of this work was to screening cotyledons , hypocotyls, epicotyls and leaves explants , ability to induction of hairy roots by *GUS* –Labeled *Agrobacterium rhizogenes*-mediated transformation by co-cultivation and direct inoculation methods.

2. MATERIALS AND METHODS

Plant materials

Mature dry seeds of *Glycine max* L. (from local market) were washed by water and surface-sterilized with 5% (v/v) sodium hypochlorite solution for 10 min., then rinsed three times in sterilized water .The seeds then soaked in distilled water for 24 h at dark condition (Paz *et al*., 2006). Each two seeds were placed on 25 ml of agar-solidified MS(Murashige and Skoog, 1962) medium in 100 ml glass bottle. The seeds were germinated in a growth room at 25±2ºC with dark conditions for 3-4 days. Then transferred to grow up under 16-h light / 8- h dark photoperiod.

Agrobacterium Preparation

GUS- labeled *Agrobacterium rhizogenes* R1000 strain (P.Urwin,Faculty of Biological Science, Leeds Univ.,UK.) was maintained on solid Luria Bertani (LB) medium (Table.1) supplemented with 50 mg L^{-1} kanamycin. A single colony was transferred to 5 ml of liquid LB medium containing the appropriate selective antibiotic, and culture was grown overnight at 28°C with 180 rpm shaking until an $OD600_{nm}$ =1.0 was reached. Before starting the transformation experiments, bacterial cells were centrifuged at 4,000 rpm for 15 min. The pellet was then resuspended in liquid co-cultivation medium (LCCM, Table.1). After adjustment to $OD600_{nm}$ of 0.5 and 1.0, the *Agrobacterium* suspension was used for co-cultivation and direct inoculation respectively .

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Table. 1 List of media used for explants tissue transformation in this Paper.

Media Composition
LB 10 g L^{-1} tryptone , 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl , 1.5 % (w/v) agar, pH 7.0
LCCM 1/4 B5 salts ,1/10 B5 vitamins, 30 g L^{-1} sucrose, 2.0 mg L^{-1} 6-BA, 0.2 mg L^{-1} IBA, 200µM L^{-1} Acetosyringone, 0.3 g L^{-1} L-Cysteine, 0.20 g L^{-1} , $Na_2S_2O_3$, pH 5.4
CCM 1/4 B5 salts ,1/10 B5 vitamins, 30 g L^{-1} sucrose, 2.0 mg L^{-1} 6-BA, 0.2 mg L^{-1} IBA, 200µM L^{-1} Acetosyringone, 0.3 g L^{-1} L-Cysteine, 0.20 g L^{-1} , $Na_2S_2O_3$, 0.4 % (w/v) agar, pH 5.4
LMXB MS salts, B5 vitamins, 30 g L^{-1} sucrose , 200 mg cefotaxime, pH 5.8
MXB MS salts, B5 vitamins, 30 g L^{-1} sucrose , 200 mg L^{-1}cefotaxime, 0.7 % (w/v) agar, pH 5.8

Explant preparation

After 7-10 days of germination, the cotyledons are ready to be cut. The cut was made between the hypocotyl and the ½ way point of the cotyledon (Paz *et al*., 2004).The seeds coat was removed from those hasn't fallen off yet. Cotyledons, primary leaves, segments of about 2-3 cm length of epicotyls and hypocotyls were placed on sterile filter papers to use as explants for inoculate with *Agrobacterium* by co-cultivation and direct inoculation methods .

Explants inoculation

Direct inoculation

All explants were inoculated with *GUS*-labeled *A. rhizogenes* R1000 $OD600_{nm}$1.0 by making 5-7scratches using sterile insulin 1.0 ml syringe after immersed it in the *A. rhizogenes* inoculum (sterile distilled water was used for control) and then transferred to dry sterile filter paper (Xue *et al*., 2006). These explants (approximately 30 wounded segments from each explants) were then placed onto 15×100-mm Petri plates containing solid co-cultivation medium (CCM ,Table .1).
Stacks of Petri dishes were wrapped with Parafilm and incubated at 25°C for 5 d in the dark .

Excess *Agrobacterium* was washed from the explants by immersing in liquid medium (LMXB ,Table.1) containing MS salts, B5 vitamins (Gamborg *et al*., 1968) and antibiotic for 30 min with 80 rpm shaking. Explants were then removed from the LMXB medium and placed on sterile filter paper and transferred onto solidified MXB medium (Table.1). After 3-4 explants per plate were placed, plates were wrapped by parafilm and incubated in growth room at 28°C under 16-h light / 8-h dark photoperiod. Explants were subcultured onto the same fresh medium at 2 weeks intervals. After the formation of hairy roots from wound sites, they were separated from the explant tissue and subcultured in the dark at 25±2°C on MXB medium containing 200 mgL^{-1} cefotaxime .

Co-cultivation

The explants which prepared above wounded carefully about 5-7 times with sterile insulin 1.0 ml syringe. Approximately 30 wounded segments from each explants were placed onto a 100×25-mm Petri dish containing 50 ml of *Agrobacterium* suspension $OD600_{nm}$= 0.5 (*Agrobacterium* with LCCM) and co-cultivated for a minimum of 30 min at room temperature. For control, 5 wounded of each explants were placed onto a 100×25-mm Petri dish containing 50 ml liquid medium (LCCM,Table.1) only without *Agrobacterium*. The explants were then removed from the suspension and placed on sterile filter paper and placed on top of a sterile 70-mm Whatman #1 filter paper on 15×100-mm Petri plates containing solid co-cultivation medium (CCM ,Table .1). Stacks of Petri dishes were wrapped with Parafilm and incubated at 25°C for 5 d in dark (Olhoft *et al*.,2007).

Agrobacterium was washed from the explants after co-cultivation by immersing in liquid medium (LMXB ,Table .1) containing MS salts, B5 vitamins and antibiotic for 30 min with 80 rpm shaking. Explants were then removed from the LMXB medium and placed on filter paper and transferred to solidified MXB medium (Table.1). Three explants per plate were placed and then the plates were wrapped by Parafilm and incubated in a growth room at 28°C under 16-h light / 8-h dark photoperiod. The explants were subcultured on the same fresh medium at 2 week intervals. After 2-3 weeks, the hairy roots were separated from the explant tissue and subcultured in the dark at 25±2°C on MXB medium containing 200 mgL^{-1} cefotaxime.

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Histochemical analysis of *GUS* expression

Histochemical *GUS* assays were conducted according to Jefferon's standard protocol (Jefferson *et al*.,1987). Hypocotyl materials were immersed in a substrate solution containing 0.1 M sodium phosphate buffer (pH 7.0), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM EDTA and 500 mgL^{-1} X-Gluc, and 0.1% Triton-X 100 at 37°C overnight. Tissue was subsequently cleared in 70% ethanol prior to visualization.

3. RESULTS AND DISCUSSION

In this study the results refer that direct inoculation of cotyledons recorded the best results , followed by hypocotyls , epicotyls and leaves (Table.2) . The results indicate that cotyledon was more amenable than other explant in producing hairy roots and recorded high values of transformation frequencies.

Table.2 Effect of direct inoculation and co-cultivation methods on production of hairy roots from explants of soybean (Glycine max L.) seedling by GUS –Labeled Agrobacterium rhizogenes strain R1000.

Inoculation methods / Explants	Direct inoculation			Co-cultivation		
	No. of survival explants	No. of explants produced H.R.	Transformation frequency (%)	No. of survival explants	No. of explants produced H.R.	Transformation frequency (%)
Cotyledons	26[a]	20	76.9	25	15	60.0
Hypocotyls	25	16	64.0	26	13	50.0
Epicotyls	24	14	58.3	23	11	47.8
Leaves	21	8	38.0	22	6	27.2

[a] **Total number of 30 explants were used / treatment for both methods.**

This is maybe due to cotyledon nature that considered the best explant to transformation , in addition to the effect of L-Cysteine , Acetosyringone and Sodium thiosulfate in LCCM and CCM media that increase the rate of transformation of explants (Olhoft and Somers, 2001; Paz *et al*., 2004;Paz *et al*.,2006) .

This suggests that L-Cysteine was increase the frequency of transformed cells by either directly affecting the soybean explant or affecting the interaction between the *Agrobacterium* and soybean explant at some stage during co-cultivation**.** L-Cysteine may increase the frequency of transformed cells by either acting as a nutritional supplement during the co-cultivation step or by acting through its thiol group (Olhoft *et al*., 2001).

Acetosyringone is one such compound used successfully to enhance transformation in various plant species in *A. rhizogenes* mediated genetic transformation. It has been known to enhance transformation efficiency due to activation of *vir* genes in *Agrobacterium* (Gelvin, 2000). Therefore we presume that the enhancement in transformation by Acetosyringone treatment may be due to activation of *vir* genes which is absolutely required for the T-DNA delivery to plant tissues (Kumar *et al*.,2006).

In this study the results showed that the both methods of infection can be utilize to produce hairy roots from the explants with varying percentage of transformation frequency, but the direct inoculation was the efficient to produce hairy roots. This perhaps due to that facilitate bacterial penetration and invasion wounds caused by needle injury on the explant.

The process of *Agrobacterium*-mediated gene transfer initiates by wounding of plant tissue, which leads to the release of phenolic compounds and monosaccharaides and subsequent triggering of the expression of *vir*-genes in *Agrobacterium* (Sheng and Citovsky, 1996). One *vir* senses plant phenolic compounds and transduce this signal to induce expression of *vir*A and *vir*G genes (Stachel *et al*., 1986). Because wounding is important for efficient plant transformation, *Agrobacterium* can sense a wounded potential host by perceiving these phenolic compounds. Infection medium also contained Acetosyringone, L-Cysteine , Sodium thiosulfate and acidic pH, these factors have already been proven to facilitate T-DNA transfer and enhance transformation efficiency (Olhoft *et al*., 2003).

In this study all explants (Fig .1 a-d) which transformed by using direct inoculation method showed different response to this method. The hairy roots were formed only from wounded regions of explants, and they were formed 2 weeks after direct inoculation (Fig.1 f-j). The average lengths of hairy roots was 7.0 cm and they were off-white in color and the culture of hairy roots was obtained after 3 weeks from

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inoculation (Fig.1.k). The control explants inoculated with only sterile water did not show induction of roots. In addition, the use of co-cultivation and direct inoculation methods showed the growth of small shoots from cotyledon explants and it was 86.6% and 93.3% respectively after one week of inoculation (Fig.1,e). This perhaps due to the use of BAP in the LCCM and CCM media (Wang and Xu ,2008) . It was observed that exogenous cytokinin application alters axillary meristem development, promotes shoot proliferation and regeneration of the cell in meristematic tissues (Zia *et al*., 2010).

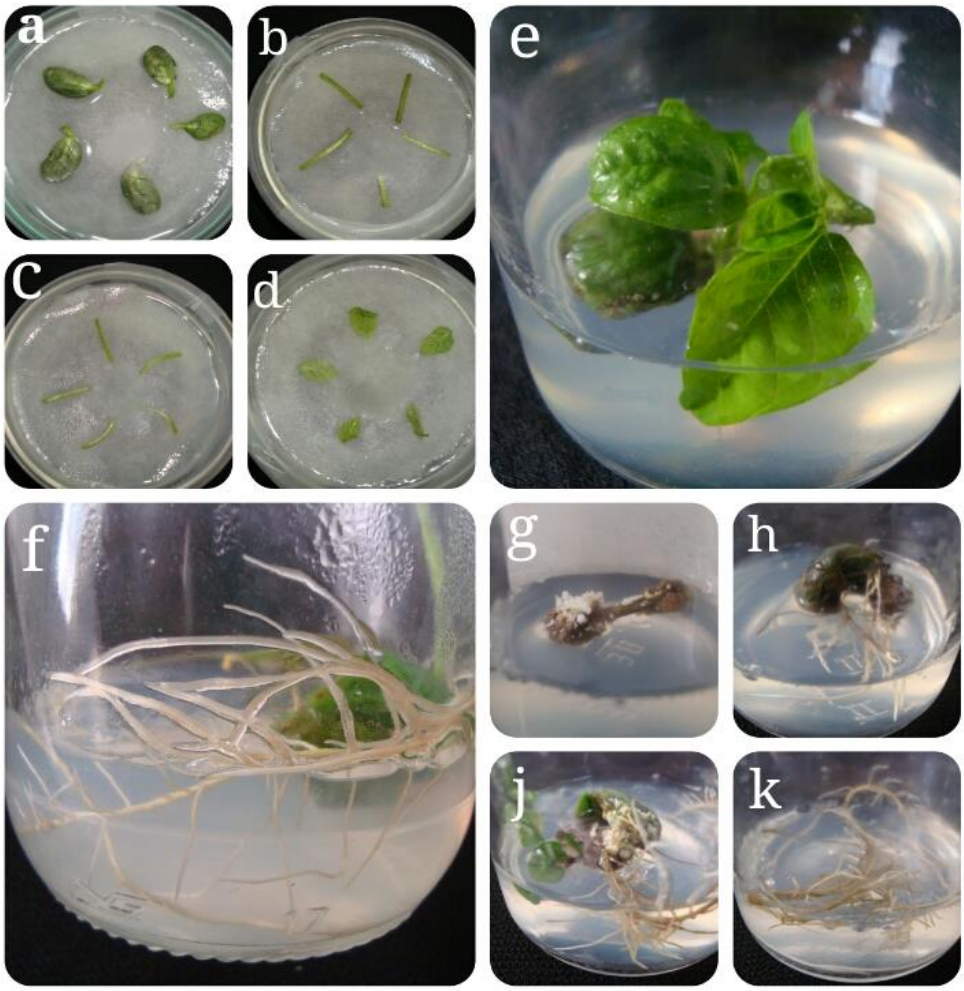

Fig.1 Transformation of soybean explants by direct inoculation method with *GUS* –Labeled *Agrobacterium rhizogenes* R1000 .
(a) Cotyledons cultured on CCM medium **(b)** Hypocotyls cultured on CCM medium **(c)** Epicotyls cultured on CCM medium **(d)** Leaves cultured on CCM medium **(e)** Formed shoot from cotyledon after one week **(f)** Hairy root formed from cotyledon that formed shoot **(g)** Hairy root initiation from hypocotyl after 10 days of inoculation **(h)** Hairy root initiation from cotyledon **(j)** two weeks after inoculation **(k)** The hairy roots after 3 weeks of culture.

In co-cultivation method the results recorded the highest level of hairy roots formation from cotyledons followed by hypocotyls , epicotyls and leaves (Table.2). The results of this method showed that after 2 weeks of inoculation the hairy roots were formed from each explants (Fig.2.a-d). The color of this hairy roots was off-white and the average length was 4.0 cm. Hairy roots culture was formed 3 weeks after co-cultivation (Fig.2.e), while no hairy roots were formed in control explants.

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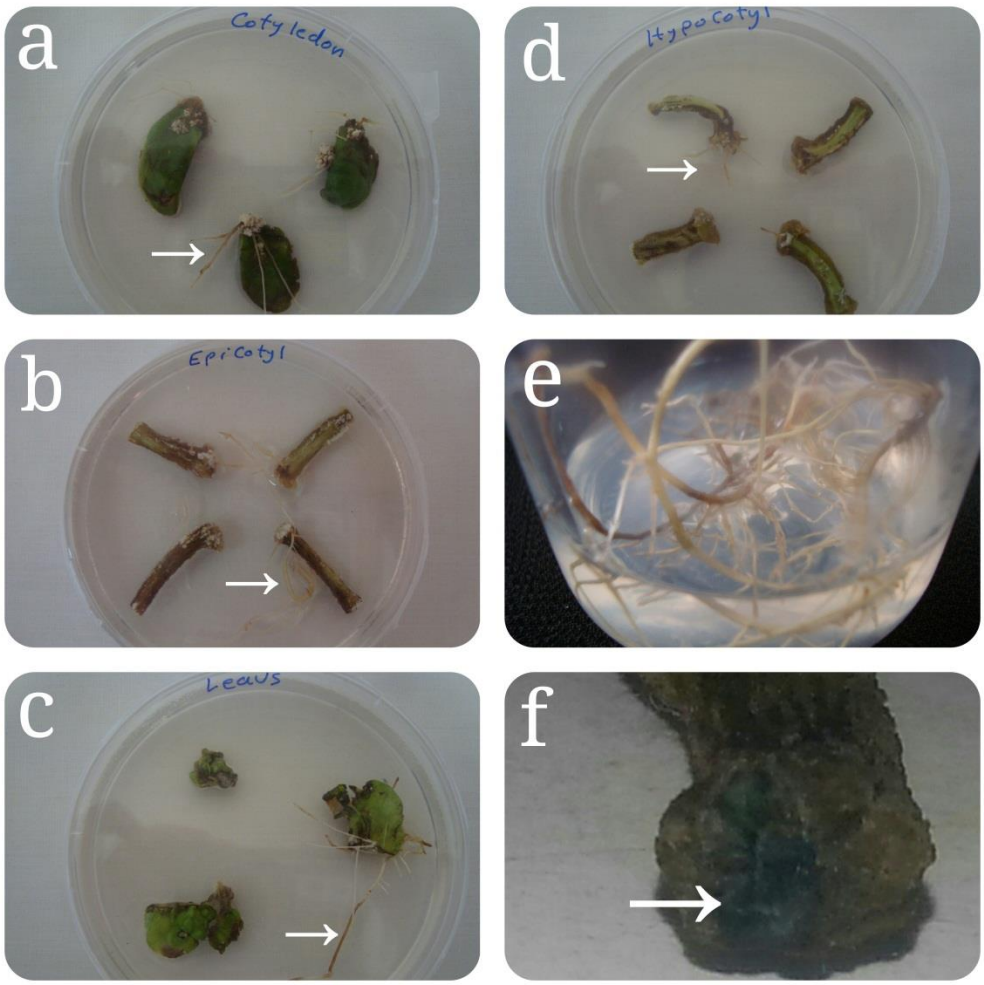

Fig.2 Transformation of soybean explants by *GUS* –Labeled *Agrobacterium rhizogenes* R1000 using co-cultivation method.
(a) Cotyledon cultured on MXB medium and numerous hairy roots (arrowed) after 2 weeks of culture **(b)** Epicotyl cultured on MXB medium and numerous hairy roots (arrowed) after 2 weeks of culture **(c)** Hairy root initiation from leaves (arrowed) after 2 week of inoculation **(d)** Hairy root initiation from hypocotyl (arrowed) after 2 week of inoculation **(e)** Accumulation of hairy roots after 3 weeks of culture on MXB medium **(f)** Histochemical *GUS* expression in hypocotyl (arrowed)

This method of explant preparation showed positive results with screening *GUS* protocol (Fig.2.f) .Staining of this transgenic tissue with X-Gluc exhibit a distinct blue color visualized with the naked eye. Blue-colored transgenic explant suggested that they expressing the blue stain constitutively confirming their transformation to express 35S *GUS* gene (AL-Mallah and Masyab,2014).

The conclusion of this study that *Agrobacterium*-mediated transformation by direct inoculation was more efficient in hairy roots induction and cotyledon was the best explant for transformation . The obtained results were not varied from those recorded by other researchers (Cho *et al*., 2000 ; Olhoft *et al*., 2007 ; Cao *et al*., 2009 ; Weber and Bodanese, 2011).

Acknowledgements

The authors thank Mr. Awab W. AL-Harbawy, Dr. Qutaiba Sh. Al-Ne'ma and Dr. Hikmat M. Masyab for extending all facilities and technical help.

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