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Optimization of Protocol for Hairy Root Induction in Tomato (*Solanum lycopersicum* L.)

S. PRABAGARAN[1*], R. MANJUNATH[2], J. ASHOKRAJ[2], R. S. SHASHIKUMAR[3], K. T. ARUNAKUMARA[4] AND K. KUMUTHA[1]

[1]*Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore 641003, India*
[2]*Division of Microbiology, Indian Agricultural Research Institute, New Delhi 110012, India*
[3]*Department of Genetics & Plant Breeding, CCS Haryana Agricultural University, Hisar 125004, India*
[4]*Department of Plant Pathology, College of Horticulture, Bidar 585403, India*
E-mail : prabakars2005@gmail.com
**Correspondence*

Abstract

Standardization of protocol for hairy root production in tomato was done using five different media with manual wounding and two levels of acetosyringone (ACS) treatment. *A. rhizogenes* strains 2364 and A4 Modified White's medium (MWM) induced more hairy roots. Manual wounding of explant with the addition of 100 μM acetosyringone in the media resulted better induction of hairy roots with all the three strains of *A. rhizogenes* studied. Molecular analysis of hairy roots was done to confirm the *agropine synthase (ags)* gene using specific primer. The primer showed the amplification of both positive control and the transformed hairy root samples with a band size of 341 kb, confirming transformation of *ags* gene to the hairy roots.

Key words : *Agrobacterium rhizogenes*, Hairy root, Acetosyringone, Tomato, *Agropine synthase* gene.

Tomato (*Solanum lycopersicum* L.) is an important Solanaceae vegetable crop with high Vitamin A and C content. They are also an excellent source of lycopene, responsible for prevention of many types of cancer. Hairy root is a plant disease caused by *Agrobacterium rhizogenes* Conn., a Gram-negative soil bacterium. When the bacterium infects the plant, the T-DNA between the TR and TL regions of the Ri-plasmid in the bacterium is transferred and integrated into the nuclear genome of the host plant. The transformation process produces a valuable by-product, hairy root, which will form at or near the site of infection. In addition, opines are produced and serve as specific food for the bacteria (1). Hairy roots grow rapidly, show plagiotropic growth, and are highly branched on phytohormone-free medium. The transformed root is highly differentiated and can cause stable and extensive production of secondary metabolites, whereas other plant cell cultures have a strong tendency to be genetically and biochemically unstable and often synthesize low levels of useful secondary metabolites (2). Most importantly, *A. rhizogenes* can transfer T-DNA from binary vectors and enable the production of transgenic plants containing foreign genes carried on a second plasmid. This property has been used to produce transgenic plants (3). The major advantage of this approach is the rapidity and technical simplicity of *A. rhizogenes* transformation. Instead of the 4-6 months-time required to regenerate transgenic plants following *A. tumefaciens* based transformation, production of Ri T-DNA transgenic roots takes only a few weeks. Thus, this technique is particularly well adapted to RNAi-based approaches to study gene function (4), performing functional promoter analyses and as a means for rapidly evaluating the complementation of plant mutants with *Agrobacterium rhizogenes*-medicated transformation candidate genes (5), when the genetic determinant of the corresponding mutation is root-determined. Research on the transformation studies in tomato is need of the hour to exploit tomato to its full potential. For the past few decades, lot of interest in tomato has aroused due to its potential as major source of lycopene, vitamin C and A. Tomato transgenic root explants can be clonally propagated for studies of endomycorrhizal colonization. It also helps to mass multiply pure culture of AM fungi. Hence the present study was designed to optimize protocol for hairy root induction in tomato.

Methods

The experiments were conducted in the laboratories of Center of Advanced Studies in Agricultural Microbiology, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India.

Treatments Subjected to Explant Segments of Tomato Prior to Co-cultivation with A. rhizogenes on Five different Tissue Culture Media

A. rhizogenes strains used	Treatments	Media used
A. rhizogenes MTCC 532	Control (without *A. rhizogenes*)	MS medium
A. rhizogenes MTCC 2364	Manual wounding	(Murashige and Skoog medium)
A. rhizogenes A4	+ *A. rhizogenes*	B5 (Gamborg's medium)
	Manual wounding + ACS 100 μ M + *A. rhizogenes*	WM (White's medium)
	Manual wounding + ACC 150 μM	MWM (Modified White's medium)
	Manual wounding + ACS 150 μM + *A. rhizogenes*	NM (Nitsch medium)

Preparation of Bacterial Culture for Co-Cultivation: A loopful of the culture from *A. rhizogenes* strains were taken from culture grown on TY medium. It was inoculated on to the 20 ml of TY broth in a sterile culture tubes. The inoculated cultures were grown overnight at 25 C in orbital shaker at 80 rpm.

Co-Cultivation. The explants (leaf and stem) of *in vitro* propagated tomato plants were aseptically transferred to the sterile filter paper and were cut into 1-2 cm bits. The explants were pricked with needle and immersed into the overnight grown pelletized *A. rizogenes* culture suspension for 5 min. The explants were taken out from the suspension and blot dried on the sterile blotting paper. After blotting, the explants were placed in hormone free basal MS medium with ACS at 100 and 150 μM concentrations without antibiotics and incubated in dark at 25 C. After 48 h, the explants were checked for the production of hairy roots and growth of bacteria on the media. If the growth were traced, then immediately the explants were transferred to a hormone free MS basal medium containing cefotaxime 500 mg/l.The explants were transferred to the fresh medium as above till the bacterial growth was completely arrested. The concentration of cefotaxime reduced in every transfer of fresh medium by 50 mg/l. After the bacterial free growth was observed, the transformed roots were sub-cultured in hormone and antibiotic free solid basal MS medium.

The transformation rate was calculated as below

$$\text{Transformation frequency} = \frac{\text{Number of explants inducing hairy roots}}{\text{Total number of explants infected with } A.\ rhizogenes} \times 100$$

Confirmation of Hairy Root Induction by A. rhizogenes Strains ags (Agropine synthase) Gene Detection by PCR

Isolation of Genomic DNA from the Transformed Hairy Roots. Hairy roots were cut into the small pieces (1–2 cm) long and ground in sterilized pestle and mortor containing 300 μl of extraction buffer (200 mM tris-HCl pH 7.5, 200mM NaCl, 25 mM EDTA and 0.5% SDS) and acid-washed sand using a pestle. The homogenate was transferred to a 1.5 ml eppendorf tube and centrifuged at 12.000 rpm for 10 min. Equal volume of isopropanol was added to the supernatant and incubated at -20 C for 20-30 min. The crude DNA was pelleted by centrifugation at 12000 rpm for 10 min. Pellets were air dried at room temperature and dissolved in 30 μl of 0.1X TE buffer (1mM Tris-HCl, pH 8.0 and 0.1mM EDTA, pH 8.0). To check the presence of genomic DNA, 10 μl of the samples were loaded in a 1.5% agarose gel in 1X TE buffer at 66 volts for 30 to 60 min. Th ethidium bromide stained gels were documented using Alpha Imager. For each PCR reaction, 2.0 μl of this DNA preparation was used as template.

Ags Gene Detection by PCR. PCR was performed to amplify T-DNA *agropine synthase (ags)* from the transformed hairy roots. The genomic DNA of *A. rhizogenes* was used as positive control. Reactions were performed in a final volume of 20 μl. The PCR mixture contained 2.0 μl of genomic DNA, 2.0 μl of 10X PCR buffer (10mM Tris-HCl pH 9.0, 50mM KCl, 1.5mM MgCl2, 0.2 mM of each of dNTPs, 1μM of

Table 1. Effect of different media on *Agrobacterium rhizogenes* 532 strain mediated hairy root induction frequency (%) in tomato explants. Values are the mean of five replicates.

Treatments	Media MS	B5	WM	MWM	NM
Control (without *A. rhizogenes*)	0	0	0	0	0
Manual wounding + A. rhizogenes	40	20	40	60	20
Manual wounding and ACS 100 μM + *A. rhizogenes*	80	60	40	80	40
Manual wounding and ACS 150 μM + *A. rhizogenes*	60	60	60	60	60
	SE			CD (0.05)	
Media	0.77			1.56	
Treatment	0.69			1.40	
M × T	1.55			3.13	

Table 2. Effect of different media on *Agrobacterium rhizogenes* 2364 strain mediated hairy root induction frequency (%) in tomato explants. Values are the mean of five replicates.

Treatments	Media MS	B5	WM	MWM	NM
Control (without *A. rhizogenes*)	0	0	0	0	0
Manual wounding + *A. rhizogenes*	40	40	40	60	20
Manual wounding and ACS 100 μM + *A. rhizogenes*	80	100	60	100	40
Manual wounding and ACS 150 μM + *A. rhizogenes*	60	80	60	100	80
	SE			CD (0.05)	
Media	0.96			1.95	
Treatment	0.86			1.75	
M × T	1.93			3.91	

forward (*Ags*-5' GCGCATCCCGAGGCGATG 3') and reverse primers (*Ags*-GGTCTGGCGATCGCGAGGA 3') and 0.15 units of *Taq* DNA polymerase. PCR amplification was performed with a program of initial denaturation at 94^{0}C for 3 min, 30 cycles of denaturation at 94 C for 1 min, annealing at 55 C for 1 min, extension at 72C for 1 min, and a final extension at 72 C for 7 min and storage at 4 C. Agarose gel electrophoresis for the detection of PCR products was done according to Sambrook et al. (6).

Results and Discussion

Transformation efficiency of *A. rhizogenes* differed with respect to host plant and media used. *A. rhizogenes* 532 strain induced maximum of 80% hairy root at 100 μM of ACS on both MS and MWM (Fig.1). However, manual wounding without ACS produced 60% hairy roots on MW Medium. The least (20%) hairy roots were observed in NM and B5 medium (Table 1). *A. rhizogenes* 1264 strain induced 100% hairy root at 100 μM of ACS treatment on both MWM (Fig. 1) and B5 medium. Also 150 μM ACS treatment had 100 per cent hairy root induction frequency in MWM (Table 2). In case of manual wounding maximum of 60% frequency was observed with MWM and the least of 20% hairy root was noticed with NM. Efficiency of transformation is known to differ with different bacterial cultures. The transformation ability of different *A. rhizogenes* strains was found to be different.

A. rhizogenes A4 strain produced maximum of

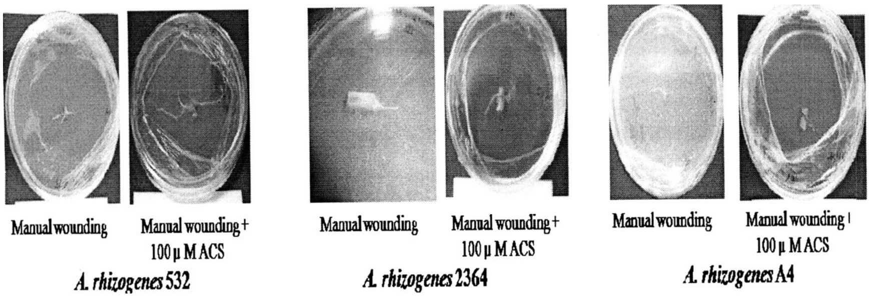

Figure 1. Hairy root induction in tomato explants using MW medium.

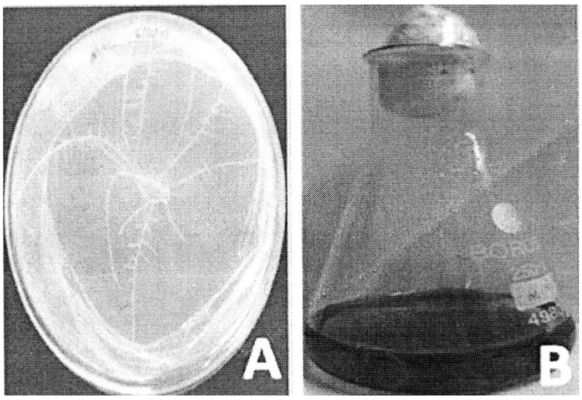

Figure 2A. 50 days of subculturing in solid MW medium. **Figure 2B.** 30 days of subculturing in liquid MW medium.

100 per cent hairy root induction at 100 μM of ACS on MS and MWM (Fig. 1). Similarly, 150 μM of ACS treatment registered 100 per cent hairy root induction in MWM medium. The induction frequency of 20 to 40% was observed with manual wounding (Table 3). Wounding enhance the accessibility of putative cell-wall binding factors to the bacterium. Acetosyringone and α-hydroxy acetosyringone induce the transcription of *vir* region. ACS has also known to enhance the transformation efficiency due to activation of *ags* gene in *A. rhizogenes* (7). Manual wounding though induced hairy roots, the frequency was observed lesser in all five media tested. Therefore, it was presumed that the enhancement in transformation be ACS treatment may be due to activation of *ags* gene which is absolutely required for the T-DNA delivery to plant tissues.

Various media were also used by several re-

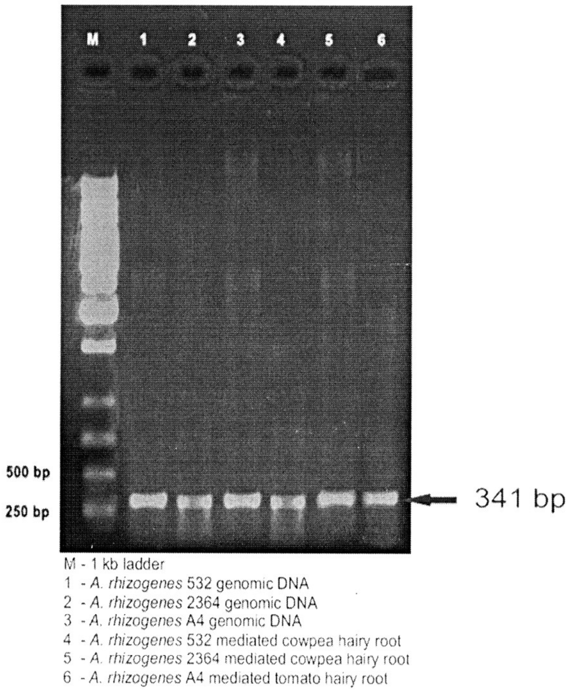

Figure 3. Confirmation of hairy root induction by *A. rhizogenes* using ags gene detection by PCR.

Table 3. Effect of different media on *Agrobacterium rhizogenes* A4 strain mediated hairy root induction frequency (%) in tomato explants. Values are the mean of five replicates.

Treatments	Media MS	B5	WM	MWM	NM
Control (without *A. rhizogenes*)	0	0	0	0	0
Manual wounding + *A. rhizogenes*	40	20	40	40	20
Manual wounding and ACS 100 μM + *A. rhizogenes*	100	40	80	100	60
Manual wounding and ACS 150 μ M + *A. rhizogenes*	60	60	80	100	40
		SE		CD (0.05)	
Media		0.91		1.84	
Treatment		0.81		1.64	
M × T		1.82		3.68	

searchers for the hairy root induction studies such as MS medium (8), M medium (9), Gamborg's B5 medium (10) for their suitability, using different host plants. In all the five media studied, no hairy root induction was observed on control (without *A. rhizogenes*). Among the treatments, 100 μM ACS addition registered higher transformation frequency of 100% in MWM with all the three strains of *A. rhizogenes*. Among the three strains evaluated. *A. rhizogenes* strain A4 performed best with tomato explants in terms of hairy root induction frequency irrespective of the media used. Efficiency of transformation is known to differ with different bacterial cultures. The transformation ability of different *A. rhizogenes* strains was found to be different. In tobacco, the performance was reported to be in the order of LBA 9402, 9340, 9365, 15834 and A4 (11). Bacterial free transformed hairy root bits from tomato were subcultured both on solid and liquid tissue culture media without growth regulators (Fig. 2). While subculturing, hairy roots showed prominent growth on all the five tissue culture media. However, profused growth of hairy roots was observed in liquid media under shaking conditions at 80 rpm.

Confirmation of Hairy Root Induction usingAgs (Agropine synthase) Gene Detection by PCR.

Molecular analysis of hairy roots was carried out to confirm the transformation of *ags* gene, PCR analysis was done using a pair of gene specific primer (forward and reverse) which ampifies the T-DNA *agropine synthase* gene. The total genomic DNA was extracted from the transformed hairy roots to observe the presence of DNA. It showed that the DNA was intact and the concentration was nearly uniform in all the samples transformed by different *A. rhizogenes* strains. This DNA was subjected to PCR amplification.

The total genomic DNA was extracted from *A. rhizogenes* and resolved in agarose gel electrophoresis.It showed that the DNA was intact and the concentration was nearly uniform in all the isolates of *A. rhizogenes*, which can be further used as positive control for PCR analysis. In PCR, the primer showed amplification which confirmed the successful transformation of T-DNA *agropine synthase* gene which produced the band of size 341 bp. (Fig. 3). The integration of Ri T-DNA leads to alterations in hormone metabolisms, transport properties and the production of opines in the transformed roots. Ri T-DNA consists of two parts T_L-DNA and T_R-DNA which is physically separated by a non-transformed DNA sequence of approximately 18 kb T_R-DNA (12). Similar primer was also used by in *Ulmus procera* to confirm the transformation (13). The studies also demonstrated the role of T_L-DNA and T_R-DNA genes on growth and alkaloid accumulation in the root clones. The present study revealed that strains of *A. rhizogenes*, growth medium and growth conditions may affect the hairy root formation and development. Hence the optimization of these factors is considered prerequisite for getting efficient transformation.

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