

Transformation of Tomato (*Lycopersicon esculentum*) by *Agrobacterium tumefaciens* using Tobacco (*Nicotiana tabacum*) Leaf Extract

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ABSTRACT

Agrobacterium mediated gene transformation method is considered as an effective method for transferring foreign genes into plants. Hence, this study was conducted to enhance foreign gene transformation efficiency of local cultivars of tomato (*Lycopersicon esculentum*) using *Agrobacterium tumefaciens* strain LBA4404 harboring binary vector (pBI121) in the presence of tobacco (*Nicotiana tabacum*) chopped leaf extract. Tomato variety, T245, was successfully regenerated from cotyledon explants cultured on Murashige and Skoog (MS) medium with 2.0 mg/l Zeatin + 0.1 mg/l Indole Acetic Acid (IAA) with 100% efficiency. Although regeneration of transformed tomato was carried out in 500 mg/l Cefotaxime and 25 mg/l levels of kanamycin Sulfate shoot emergence was not occurred. Hence, as positive control tobacco (*Nicotiana tabacum*) leaf explants were cultured on kanamycin after inoculation in *Agrobacterium* in order to examine whether pBI121 function positively. The culture exhibited many shoots indicating possible true tobacco transformants as compared to the negative control. Hence, an experiment was conducted using tomato explants inoculated with *Agrobacterium* together with chopped tobacco leaf extract and the explants were cultured on 25 mg/l level of kanamycin. The results showed successful transformants with 40% efficiency. Further experiments are necessary to confirm the transformation event by β -glucuronidase (GUS) histochemical staining. This system is possible to be applied to insert the foreign genes into local tomato varieties using low cost transformation enhancing agents such as tobacco leaf extract.

Key words : *Agrobacterium tumefaciens*, Cotyledons, kanamycin, Tomato, Transformation

INTRODUCTION

Tomato (*Lycopersicon esculentum*) is one of the popular vegetables in Sri Lanka. Yield and the quality of tomato are affected by many factors including pest and diseases, unfavorable weather conditions and post-harvest handling. The crop is highly subjected to post harvest losses during storage and transportation. Therefore crop improvement activities have been mainly directed towards the development of varieties with high yield, pest and disease tolerance and resistance to long term storage.

Investigations on use of biotechnology for the improvement of *Lycopersicon esculentum* have been carried out extensively in recent years. Gene transformation is considered as the only method used to introduce alien genes with favorable characters. One of the most effective means of gene transfer into dicotyledonous plants has been reported as the *Agrobacterium tumefaciens* mediated natural transformation. It is well known fact that genes located within the border sequence of the *Agrobacterium* Ti plasmid are inserted into the genome of the host by random integration. Utilization of this mechanism for gene transfer requires both, susceptibility to infection by *Agrobacterium* and the ability to regenerate plants from individually transformed cells via tissue culture (Kottearachchi et al., 2000).

A number of factors contribute to the overall production efficiency of the *Agrobacterium* gene transformation. Up to now, the researchers have studied some factors affecting *Agrobacterium*-mediated transformation efficiency such as co-cultivation duration, plant genotype, stage of explants, role of phenolic compounds, vector construct, *Agrobacterium* strain, bacterial density, infection temperature, and

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This paper describes a successful approach to obtain regeneration of tomato plants from cotyledon explants and transformation of tomato by *Agrobacterium tumefaciens* method using tobacco chopped leaves as an alternative approach for the use of acetosyringone

This study was conducted at the tissue culture laboratory of the Department of Bio-technology, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka from March 2010 to December 2010

Seeds of mature tomato, variety 'T245' were sterilized by shaking in 15% clorox for 20 min. The seeds were cultured on MS (Murashige and Skoog, 1962) medium supplemented with 3% (w/v) sucrose, and 0.75% agar under 8 hr photoperiod conditions provided by white fluorescent lamp. Cotyledon segments of 7-10 day old germinated seedlings were used as the explants. Cotyledon pieces in 0.25 cm² size were excised and cultured in MS medium with 2.0 mg/l Zeatin and 0.1 mg/l Indole Acetic Acid (IAA) (Moghaieb et al., 2006). Plant regeneration efficiency was calculated using the formula, Plant regeneration efficiency (%) = Number of explants with shoots X 100/Number of explants cultured (Prematilake et al., 2002).

Agrobacterium tumefaciens strain LBA4404 harboring the pBI121 binary vector (Jefferson et al., 1987, Figure 1) was obtained from the Genetic Engineering Research Centre, Cairo University, Egypt. Single colony of *Agrobacterium tumefaciens* was grown in 15 ml liquid LB (Luria-Bertani) broth supplemented with 100 mg/l kanamycin sulfate. Cultures were incubated at room temperature overnight in the horizontal shaker at 150 rpm.

One set of explants were directly soaked in overnight culture of *Agrobacterium* solution for 10 min. Another set of explants was pre-conditioned by culturing intact cotyledon or leaf tissue in the same regeneration medium without any antibiotics for two days prior to the inoculation by *Agrobacterium* as described by Prematilake et al., 2002.

Agrobacterium solution to ensure the *Agrobacterium* carrying binary vector function positively by integration of T-DNA segment. As the treatment 'A' did not produce transformants, treatment 'C' was designed with tomato cotyledon explants soaked in overnight culture of *Agrobacterium* solution supplemented with tobacco chopped leaf extract. In the treatment 'D' tomato cotyledon explants were soaked in overnight culture of *Agrobacterium* solution supplemented with 50 µM acetosyringone. The explants were blotted on sterilized filter paper to remove excess bacteria and placed on the regeneration medium contained with MS medium + 2.0 mg/l Zeatin + 0.1 mg/l IAA for two days in the dark at 27°C for cocultivation purpose.

After two days of co-cultivation, the *Agrobacterium* infected explants were transferred into petri plates containing the same regeneration medium supplemented with 500 mg/l of Cefotaxime (to eliminate bacterial carry over) and 25 mg/l level of kanamycin sulfate. As replicates three petri plates, each with 8 explants were maintained for all treatments separately and transformation efficiency was calculated using the formula described by Premathilake et al., 2002. As a negative control, untransformed tomato cotyledons and tobacco leaf explants were cultured on regeneration media supplemented with kanamycin.

RESULTS AND DISCUSSION

Regeneration of *Lycopersicon esculentum*

Cotyledons were able to produce shoots within 2-3 weeks and by the end of 4th week regeneration efficiency of cotyledons was 100%. These results were similar to the results obtained by Moghaieb et al., 2006 using cotyledon explants of *Lycopersicon esculentum*. However the 'T245' variety used in this study did not regenerate with hypocotyl explants as described by Moghaieb et al., 2006.

Regeneration of Transformed Tomato

Negative controls cultured with both tobacco and tomato explants on 25 mg/l kanamycin level could not produce any shoot (Figure 2). It may be due to the fact that kanamycin available in the medium could not be detoxified as *Agrobacterium* strain carrying binary vector with kanamycin resistant gene was not inoculated. Transformed tobacco leaf explants cultured on MS medium + 2.0 mg/l Zeatin + 0.1 mg/l IAA with 25 mg/l kanamycin initiated shoots by 12 days after culturing and the results obtained by 4th week after culturing are presented in Table 1.

Table 1: Regeneration efficiency of different explants after inoculation with *Agrobacterium* cultured on different kanamycin levels at the 4th week

Treatment for explant	Method of culturing	Transformation efficiency (%) at 25 mg/l kanamycin
A (Tomato cotyledons)	P	0
	N P	0
B (Tobacco leaf pieces)	P	68.5
	N P	57
C (Tomato cotyledons + TCL)	P	40
	N P	0
D (Tomato cotyledons + AS)	NP + AS	48

P: Preconditioned, NP: Non preconditioned, AS: Acetosyringone, TCL: Tobacco chopped leaf extract.

Premathilake et al., 2002 have observed that tomato explants co-cultivated after pre-conditioning for two days produced true transformants. However, none of the preconditioned tomato explants inoculated by *Agrobacterium*, induced shoots from any medium supplemented with kanamycin in the absence of acetosyringone or tobacco chopped leaf extract.

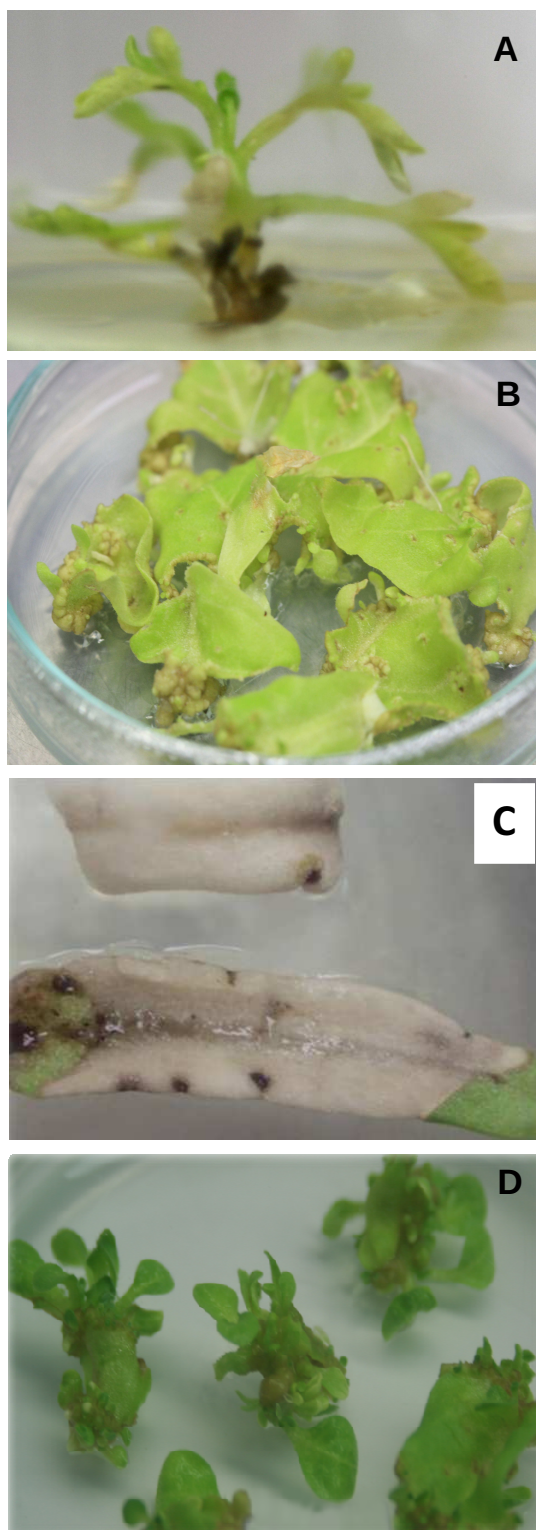


Figure 2. Transformed and non-transformed tomato and tobacco plantlets

A: 2 month old transformed tomato grown at 25 mg/l kanamycin, B: Regeneration of transformed tobacco at 25 mg/l kanamycin (positive control), C:

Non-transformed tomato cotyledon explants at 25 mg/l kanamycin (Negative control), D: Regeneration of transformed tomato (4 week old) with the help of tobacco chopped leaf extract at 25 mg/l kanamycin

None of the transformed tomato explants in treatment 'A' grew on the selective medium supplemented with 25 mg/l kanamycin. However according to the results of the tobacco explants it can be assumed that T-DNA segment of pBI121 transformation has occurred effectively with tobacco and neomycin phosphotransferase gene (NPTII) in T-DNA has expressed properly and detoxified the kanamycin in the medium. Therefore, an experiment (treatment C) was conducted to culture tomato leaf explant treating with tobacco chopped leaf extract in *Agrobacterium* inoculum. According to the results, twelve days after culturing several explants of treatment 'C' exhibited shoot initiation at 25 mg/l level of kanamycin (Table 1). This incident might have occurred due to the induction of virulence genes of *Agrobacterium* stimulated by the chemicals of tobacco extract. The first transgenic plants expressing engineered foreign genes were reported as tobacco plants (Horsch et al., 1984). In this experiment, tobacco was used as positive control and addition of chopped tobacco leaf extracts also seems to have induced virulence genes in *Agrobacterium* supporting tomato transformation. The *Agrobacterium* contains the binary vector plasmid pBI121 and its T-DNA region contains a plant expressible bacterial derived neomycin phosphotransferase II (NPT II) gene which upon transfer, genome integration and expression in plant tissue, conferred resistance to the antibiotic kanamycin. Therefore, the tomato shoots derived from the medium containing 25 mg/l kanamycin (Figure 2) indicated that at least NPTII gene sequence have been transferred into tomato tissues by using the *Agrobacterium* strain.

As expected, the transformed tomato explants co-cultured with acetosyringone was able to regenerate. According to the previous studies

transformation of *Lycopersicon esculentum* has been observed only after treating with acetosyringone into the *Agrobacterium* inoculums or co-cultivation medium (Moghaieb et al., 2006 and Ankenbauer and Nester, 1990). However, our results indicate that addition of tobacco chopped leaf extract into *Agrobacterium* inoculum could be a cost effective method for transfer foreign genes into *Lycopersicon esculentum*.

CONCLUSIONS

Tomato was successfully regenerated using cotyledon explants cultured on MS medium with 2.0 mg/l Zeatin + 0.1 mg/l IAA giving 100% efficiency. Tobacco explants inoculated with *Agrobacterium* were able to grow under 25 mg/l kanamycin while non-inoculated tobacco explants were not able to expand even suggesting the possible transformation. Few tomato plantlets were regenerated from the cotyledon explants that were inoculated with *Agrobacterium* together with tobacco leaf extract when they were subjected for pre-conditioning and grown under 25 mg/l kanamycin. Hence, addition of fresh tobacco chopped leaf extract could be considered as low cost option instead of addition of acetosyringone when foreign genes are transformed into tomato. Further experiments are necessary to conduct on assaying β -glucuronidase enzyme activity in order to confirm the transformation of T-DNA segment.

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