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Effect of explants, bacterial cell density and overgrowth-control antibiotics on transformation efficiency in tomato (*Solanum lycopersicum* L.)

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Abstract

Improved protocol for *Agrobacterium* mediated transformation of tomato cultivar, "Dhanshree" was developed by optimizing various parameters that affect transformation efficiency. In the present investigation, *Agrobacterium* strain EHA 105 harboring a pBI121: *gus* gene construct was used for transformation. The kanamycin concentration was standardized and 50 mg/L was found to be optimum based on lethal effect to the explants. Effect of varying concentration of *Agrobacterium* on the transformation efficiency of cotyledon explants revealed that the concentration of 0.2 at OD_{600} was optimum. Cotyledons proved to be better for transformation as compared to hypocotyls and leaf explants. Highest transformation efficiency was obtained in 7-14 days old cotyledon which was precultured for one day on the MS medium containing 2 mg/L zeatin and 0.2 mg/L IAA. It was then co-cultivated with *Agrobacterium* for 3 days on the same medium composition used for preculture. Subsequently the explants were transferred to selective shooting medium supplemented with 50 mg/L kanamycin, 250 mg/L cefotaxime and 250 mg/L carbenicillin. These explants were maintained for 6-8 weeks which resulted in more than 12 % transformation efficiency as judged by *GUS* assay technique.

Key words: *Agrobacterium*, *GUS*, transformation, *Solanum lycopersicum* L.

Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most important vegetable crop and used as a genetical model for introduction of agronomically important traits. There is a great potential for genetic manipulation of tomato to enhance productivity through increasing pest and disease resistance, environmental stress tolerance and to study gene function and regulation. *Agrobacterium tumefaciens* mediated transformation is the most commonly used method for obtaining transgenic plants. This method have remarkable advantages over direct transformation methods, including preferential integration of defined T-DNA into transcriptionally active region of chromosome (Olhoft *et al.*, 2004) with exclusion of vector DNA (Fang *et al.*, 2002), reducing the copy number of the transgene (Hansen *et al.*,1997). In addition, it is a single cell transformation system and avoids the obtainment of mosaic plants, which are more frequent when direct transformation is used (Enriquez *et al.*, 1998). *Agrobacterium* mediated transformation method, promote minimal rearrangement of the transgene and yield good transgenic plant fertility (Opabode, 2006). Introduced traits have been found to be stable over at least five generation during cross breeding and seed increase in genetically engineered tomato (Gasser and Fraley, 1989). Koorneef *et al.* (1987) emphasized the use of *Agrobacterium* mediated transformation in tomato because of relative simplicity of the transformation.

The first report of *Agrobacterium* mediated tomato transformation was by McCormick *et al.* (1986). Since then there have been many attempts of tomato transformation for a variety of purposes, including characterization of gene function, production of insect and disease resistant plants, herbicide tolerance, improved fruit quality, delay in fruit ripening and production of foreign proteins (Sharma *et al.*, 2009).

Inspite of the successes of tomato transformation most of the procedure relied on cumbersome either feeder layers, time consuming media formulations or successive subculture that produced significant variability between genotype. Limitation with this system includes: poor shoot regeneration capacity from leaf explants in tomato (McCormick *et al.*, 1986), relatively low transformation rate with fresh explants (Fillatti *et al.*, 1987), genotype varies in their response to specific treatment and standardization of various procedure is incomplete (Park *et al.*, 2003). The absence of highly efficient regeneration and transformation procedure is major obstacle in application of gene transformation technology to economically important tomato cultivars (Velcheva *et al.*, 2005).

The transfer of T-DNA and its integration into the plant genome is influenced by several *A. tumefaciens* and plant tissue specific factors. These includes plant genotype, explants used, pH, co-cultivation medium, *Agrobacterium* cell density, bacterial strain, addition of *vir* gene inducing synthetic phenolic compounds, culture media composition, regeneration and co-cultivation condition, tissue damages, suppression and elimination of *A. tumefaciens* infection after co-cultivation (Gao *et al.*, 2009).

Keeping in view these factors, aim of our study was to develop efficient and reproducible tomato transformation system by simplifying media conditions, eliminating frequent media changes and systematically applying these conditions to tomato cultivar to achieve high transformation frequency.

Materials and methods

Plant material: Seed of *Solanum lycopersicum* cultivar "Dhanshree" were collected from All India Coordinated Tomato Improvement Project, MPKV, Rahuri. Seeds were surface sterilized in 4 % sodium hypochlorite solution followed by washing several times with sterile distilled water. Seeds were germinated on a MS inorganic salt medium (Murashige and Skoog, 1962) containing 30 g/L sucrose, pH 5.8 and solidified using 8 g/L agar and kept at 25 °C with a 16 hour light period and 8 hour dark period.

Bacterial strains and plasmids: A recombinant binary vector pBI121 was mobilized into *A. tumefaciens* strain EHA 105 by freeze thaw method (Jyothishwaran *et al.*, 2007). To investigate the influence of *Agrobacterium* density on transformation efficiency and transient *gus* expression, the *Agrobacterium* strain was grown for 48 hours in a LB (Luria Bertani) medium containing 50 mg/L kanamycin and 10 mg/L rifampicin at 28 °C on a rotary shaker (200 rpm) until an OD_{600} =1 was obtained. Bacterial suspension were centrifuged at 8000 rpm for 10 min. Bacteria were resuspended in LB medium without antibiotics and diluted to OD_{600} = 0.2, 0.4, 0.6, 0.8 and used for co-cultivation. Cefotaxime and carbenicillin were tested for efficiency of controlling *Agrobacterium* overgrowth. 250 mg/L or 500 mg/L cefotaxime, 250 mg/L or 500 mg/L carbenicillin, 250 mg/L cefotaxime plus 250 mg/L carbenicillin or 500 mg/L cefotaxime plus 500 mg/L carbenicillin were added into shoot initiation, shoot elongation and rooting medium.

Determination of suitable kanamycin concentration in selection medium: To determine suitable kanamycin concentration in selection medium, hypocotyls explants were precultured and consequently transferred onto MS + 2 mg/L zeatin, 0.2 mg/L IAA, 2 g/L clarigel, pH 5.8 medium containing kanamycin at 0, 30, 50, 75 and 100 mg/L. Ten hypocotyls were placed in each dish and replicated three times for each concentration. Over a period of three weeks, the number of explants survived were counted and recorded in each week.

Transformation protocol: Cotyledons and hypocotyls were obtained from three age groups of seedlings (7-14 days, 14-21 days and, above 21 days old seedlings) and leaves were obtained from two age group of seedlings (14-21 days and, above 21 days old seedlings). Approximately the top half of hypocotyls along with cotyledons and leaves were detached from the seedling and placed in MS liquid medium. The excision of cotyledon from hypocotyls was done extremely carefully to prevent the bruising of tissue. The top 1 cm hypocotyls were used. The cotyledon and leaf pieces were placed upside down on preculture medium (MS + 2 mg/L zeatin, 0.2 mg/L IAA, 2 g/L clarigel, pH 5.8). Hypocotyls were placed horizontally on preculture medium and incubated overnight in the dark at 27 °C temperature. The explants were removed from preculture medium, dipped in an *Agrobacterium* culture for 3 min, blotted dry on blotting paper and recultured on the fresh preculture medium for three days. Co-cultivated explants were transferred to selective shoots regeneration medium (MS+ 2 mg/L zeatin; 0.2 mg/L IAA, 50 mg/L kanamycin; 250 mg/L, cefotaxime, 250 mg/L carbenicillin). One hundred fifty explants were grown on selective medium and replicated thrice. Explants were sub cultured after 3 weeks and allowed to grow for 6-8 weeks. Those shoots greater than 2 cm in length were excised and transferred to rooting medium (MS without any growth regulators). One set was kept as a control without co-cultivation.

***GUS* assay:** The histochemical assay for *gus* gene expression study was performed by established methods (Jefferson *et al.*, 1987). Kanamycin resistant plant produced *via Agrobacterium* mediated transformation and control plants were selected and subjected to histochemical *GUS* assay.

Result and discussion

The minimum lethal concentration to kill all the explants in three weeks was found to be 50 mg/L kanamycin. Thus, 50 mg/L kanamycin was used as a selection pressure to select transgenic plant (Table 1). Hu and Phillips (2001) also suggested that 50 mg/L kanamycin concentration was optimal for tomato regeneration and selection procedure. Among the various cell density of *Agrobacterium* tested on two explants, cotyledons and hypocotyls, the highest transformation efficiency (12.22 %) was obtained in cotyledons when *Agrobacterium* concentration OD_{600} = 0.2 was used (Table 2 and Fig. 1). Similarly highest transformation efficiency of 9.55 % was obtained with this cell density when hypocotyls were used as explants (Table 2 and Fig. 1). These results are in agreement with earlier reported results where it was reported that *Agrobacterium* cell density during infection was found to influence transformation efficiency and obtained 20.87 % transformation efficiency using OD_{600} 0.2, among the *Agrobacterium* concentration of OD_{600} 0.2, 0.5 and 1.0 (Qiu *et al.,* 2007).

Age of explants affects the transformation efficiency. To study the effect of explants age on transformation efficiency, three explants of various stages of development were used as transformation targets. Cotyledon explants obtained from 7-14 days old seedling showed highest transformation frequency of 12.22 % (Table 5 and Fig. 2). Efficiency of transformation decreased as age of explants increased. The transformation efficiency was decreased to 9.44 % with cotyledon explants obtained from 14-21 days old seedling and 7.33 % with cotyledon explants from above 21 days old seedling. On an average 9.66 %, transformation frequency was obtained with cotyledon explants (Table 5 and Fig. 2). Patil *et al.*

Table 1. Number of explants surviving on medium with varying concentration of kanamycin

Treatment	Number of explants survived on 7th day	Number of explants survived on 15h day	Number of explants survived on 21th day
MS+2mg/L zeatin+0.1mg/L IAA	30 (100 %)	30 (100 %)	30 (100 %)
MS+2mg/L zeatin+0.1mg/L IAA +30mg/L kan	12 (40 %)	7 (23.33 %)	4 (13.33 %)
MS+2mg/L zeatin+0.1mg/L IAA + 50mg/L kan	8 (26.66 %)	3 (10 %)	0 (0 %)
MS+2mg/L zeatin+0.1mg/L IAA +75mg/L kan	6 (20 %)	0 (0 %)	0 (0 %)
MS+2mg/L zeatin+0.1 mg/L IAA+100mg/L kan	5 (16.66 %)	0 (0 %)	0 (0 %)

30 hypocotyls were used in each treatment

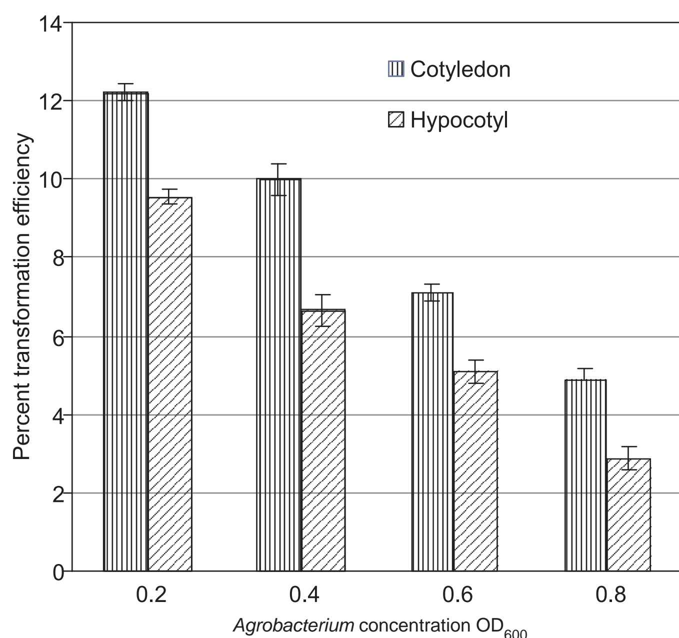

Fig. 1. Effect of *Agrobacterium* concentration on the transformation efficiency of tomato

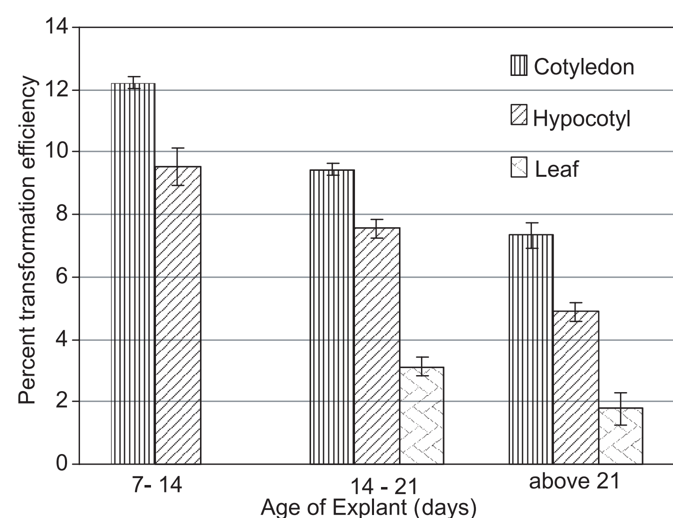

Fig. 2. Effect of age of explants on the transformation efficiency of tomato

Table 2. Effect of *Agrobacterium* concentration on the transformation efficiency of tomato

Agrobacterium Concentration (OD_{600})	Cotyledon explants Transformation efficiency (%) ± S.E.	Hypocotyl explants Transformation efficiency (%) ± S.E.
0.2	12.22±0.2*	9.55±0.2*
0.4	10.00±0.4*	6.66±0.4*
0.6	7.11±0.2*	5.11±0.3*
0.8	4.88±0.3*	2.88±0.3*
C.V.	3.045 %	
S.E.	0.382	
LSD (*P*=0.05)	0.82	

150 cotyledons and hypocotyls were used for each replication; The data are mean ± SE from three replicates, *Significant difference at *P*=0.05.

(2002) also observed strong impact of plant age in transformation of tomato.

Hypocotyls and leaf explants have been reported to be inferior for transformation as compared to cotyledons (Park *et al.*, 2003). Results of this study show that cotyledon is the best explant for transformation but hypocotyls was not found to be inferior explants and could also be used in transformation. A transformation frequency of 9.55 % was obtained using hypocotyls explants obtained from 7-14 days *in vitro* grown seeding. Transformation frequency decreased to 7.55 and 4.88 % when hypocotyls explants obtained from 14-21 days and above 21 days old seeding used, respectively (Table 5 and Fig. 2).

Transformation efficiency with leaf explants found to be very low. Only a 3.11 % of transformation efficiency was recorded with leaf explants obtained from 14-21 days old seeding. Transformation efficiency further decreased to 1.78 % with leaf explants obtained from seedlings above 21 days old (Table 5 and Fig. 2). Thus, it is proved that leaf explants was inferior for transformation.

Agrobacterium overgrowth frequency was higher when cefotaxime and carbenicillin were used individually. It was found that combination of 250 mg/mL cefotaxime and 250 mg/mL carbenicillin is very effective to eliminate *Agrobacterium* during transformation in cotyledon and hypocotyls explant and did not adversely affect regeneration frequency (Table 3 and 4). Ling *et al.* (1998) also reported that combinations of antibiotic were

Table 3. Effect of different antibiotics used for *Agrobacterium* overgrowth control on the regeneration and transformation of tomato cotyleon explants

Concentration of antibiotics	Cefotaxime (mg/L)		Carbenicillin (mg/L)		Cefotaxime + Carbenicillin (mg/L each)		No antibiotic
	250	500	250	500	250	500	
No *Agrobacterium*, regeneration frequency (%)	84.66	83.33	88	80.66	82.66	76	91.33
Agrobacterium overgrowth frequency (%) at 14 d	76	59.33	58	54.66	0.0	0.0	100
Agrobacterium overgrowth frequency (%) at 28 d	100	90	100	73.33	0.0	0.0	100
Transformation efficiency (%)	00	2.66	00	3.33	12.22	9.33	00

150 cotyledons were used for each replication; The data are mean from three replicates

Table 4. Effect of different antibiotic treatment for control of *Agrobacterium* overgrowth on the regeneration and transformation efficiency of tomato hypocotyl explants

Concentration of antibiotics	Cefotaxime (mg/L)		Carbenicillin (mg/L)		Cefotaxime + Carbenicillin (mg/L each)		No antibiotic
	250	500	250	500	250	500	
No *Agrobacterium*, regeneration frequency %	82.66	82	82.66	76.66	79.33	74	87
Agrobacterium overgrowth frequency (%) at 14 d	67.33	62	50.66	42	0.0	0.0	100
Agrobacterium overgrowth frequency (%) at 28 d	100	86	100	67.33	0.0	0.0	100
Transformation efficiency (%)	00	2.66	00	3.33	9.55	7.33	00

150 hypocotyls were used for each replication; the data are mean from three replicates

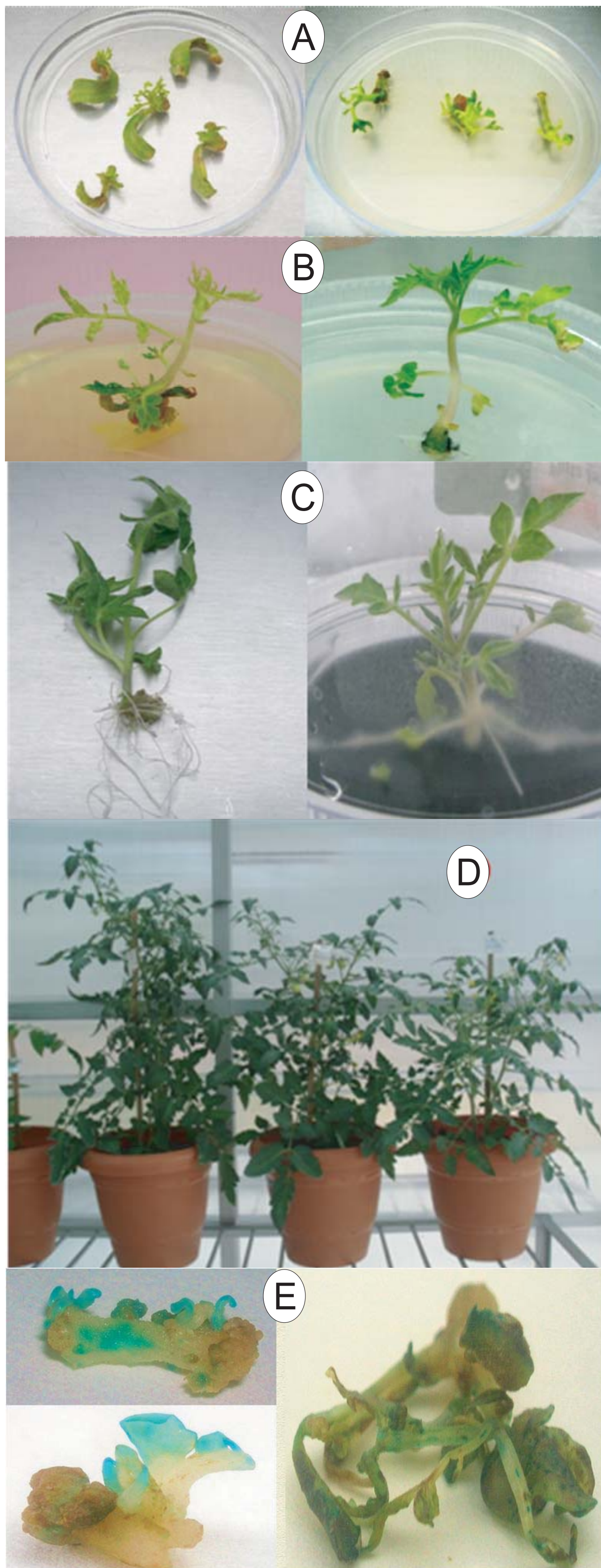

Fig. 3. A: Direct multiple shoot induction of incubation on selective shoots regeneration media; B: Putatively transformed shoots; C: Rooting of an elongated shoot on MS Medium; D: Transformed tomato plants established in biocontainment green house without; E: Histochemical *GUS* assay in different transgenic tomato parts

Table 5. Transformation efficiency (%) of three explants of tomato cultivar "Dhanshree"

Treatment	Cotyledon explant	Hypocotyl explants	Leaf explant
7-14 days old seedling	12.22± 0.2*	9.55± 0.6	-
14-21 days old seedling	9.44 ± 0.2	7.55± 0.3	3.11± 0.3
Above 21 days old seeding	7.33± 0.4	4.88± 0.3	1.78± 0.5
C. V. %		4.21	
S. E.		0.45	
C. Difference		0.96	

150 cotyledons and hypocotyls were used for each replication; the data is mean ± SE from three replicates, significant difference at 5 % level

effective in stimulating callus formation, shoot regeneration and eliminating *Agrobacterium*.

Transgenic plants when stained with X-gluc showed *GUS* activity in leaves and stems as evident by blue stain (Fig. 3E). Plant parts from control showed no detectable *GUS* activity when stained with X-gluc. About 88 % of these kanamycin resistant regenerants were transformed.

From these experiments, a reproducible and highly efficient transformation method was developed for tomato transformation. This transformation protocol yielded transgenic tomato explants expressing *gus* gene and also *npt*II gene which is used as selectable marker for transformation. *GUS* assay analysis confirmed that the transgene was successfully integrated into kanamycin resistant plants. This transformation protocol proved to be simple, repeatable and does not require any callusing phase, and may become general means of transforming tomato.

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