



## Factors affecting transformation efficiency of shoot apices of *Gossypium arboreum* and *Gossypium hirsutum* cultivars with *Agrobacterium tumefaciens*

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### ABSTRACT

In this study, two cotton cultivars viz. LD 694 (*Gossypium arboreum* L.) and LH 2076 (*Gossypium hirsutum* L.) were used to investigate factors influencing efficiency of transformation from the shoot apices of aseptically germinated seedlings for the *Agrobacterium* mediated transformation using the *Agrobacterium* strain GV3101 having the plasmid pPZP200 vector, carrying *cryIac* gene. The objective of study was to develop genotype independent *Agrobacterium* mediated transformation method. Antibiotics sensitivity assay showed that Kanamycin @ 50mg/l inhibited the regeneration of shoot tips in both LD 694 and LH 2076 genotypes and Cefotaxime @ 250mg/l check the bacterial growth. For *Agrobacterium* mediated transformation, a dilution of 500 µl /25ml of water using dip method and co-cultivation for 72 hours were resulted in better infection and survival of agroinfected explants with GV3101 strain in both genotypes. Average survival of 11.42% and 13.51% was recorded for *Agrobacterium* transformed shoots on selection medium supplemented with 50 mg/l Kanamycin and 250mg/l Cefotaxime in LD 694 and LH 2076 genotypes, respectively. GUS expression of transformed tissues ranged from 5.5-12.0 % in shoot tips. PCR analysis confirmed the presence of *Cry Iac* gene in putative transformed tissues.

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### Introduction

Cotton (*Gossypium* spp.) is an excellent natural source of textile fiber and is a high value commercial crop. Over 180 million people are associated with the fiber industry that produces 20 to 30 billion dollars worth of raw cotton (Ozyigit and Gozukirmizi, 2009). Both diploid (*Gossypium arboreum*) and tetraploid (*G. hirsutum*) cultivars are cultivated in different regions of India and are considered as important crop plants of the country. Because of its high economic value considerable attention has been paid to improve cotton plant by conventional plant breeding methods, it is time-consuming and commercialization of new cotton varieties often takes 6 to 10 years (Sheidai et al., 2008).

Additionally, indiscriminate use of pesticides to control insect-pest has led to series of consequences like, insecticide resistance, pest resurgence, outbreak of secondary pests, harmful residual effects, imbalances in natural ecosystem and higher production costs (Sanghera et al., 2011). Besides, genetic improvement of cotton through conventional means is limited due to many factors like absence of necessary variation, especially resistance against pests and diseases and its compatibility associated with pest resistant wild species. Plant tissue culture techniques provide an alternative means of improvement obtaining somaclones, induced variants, somatic hybridization and doubled haploids to develop inbred lines or for introducing genes of interest against insects and different diseases from unrelated sources such as microbes through genetic engineering (Zhang and Zhao, 1997, Kumar, 2003, Sanghera et al., 2009a). Genetic transformation of cotton has been a challenge due to genetic variability of cultivars and

differences in their responses to the in vitro culture and transformation procedures

Use of *Agrobacterium* as a vector is technically simple and gene transfer are often low copy, permanent and heritable. Therefore, *Agrobacterium* mediated transformation is the most widely used method for genetic transformation in plants. Introduction of foreign genes in elite genotypes is limited by the genotype specific nature of gene transfer in cotton (Jin et al., 2006). Cocker genotypes, which are amenable for regeneration in vitro by somatic embryogenesis, are widely used in genetic transformation experiments (Finer and McMullen, 1990). However, alternate procedures to transform non-cocker genotypes have been reported (Satyavathi et al., 2002, Trolinder et al., 2006, Kategri et al., 2007, Nandeshwar et al., 2009). In many cases, the production of transgenic plants is prevented due to the inability to regenerate plants from those tissues susceptible to transformation. So, the present study was undertaken to optimize the factors influencing *Agrobacterium* mediated transformation of two newly developed Indian cotton LD 694 (*Gossypium arboreum*) and LH 2076 (*Gossypium hirsutum*) cultivars using shoot apical meristem explants.

### Materials and Methods

#### Seed germination and cultivation of sterile seedlings

Seeds of two cotton cultivars viz. LD 694 (*G. arboreum* L.) and LH 2076 (*G. hirsutum* L.) used as source material were obtained from Cotton Section, Department of Plant Breeding and Genetics, and were delinted by using concentrated commercial H<sub>2</sub>SO<sub>4</sub>. The seeds were continuously stirred in H<sub>2</sub>SO<sub>4</sub> by wooden rod for 5~10 min until the shiny surface of seeds appeared. Some water was then added and stirred for a

few seconds. The seeds were thoroughly washed five times with tap water to remove the acid completely, left in a beaker of water for few minutes, after which those floating on the water surface were discarded. Plump, mature seeds were chosen and washed in a solution containing a few drops of Tween 20 to which water was added, vigorously shaken and then thoroughly washed thrice by autoclaved water. Surface sterilization of seeds was done by using HgCl<sub>2</sub> (0.1%) + Bavistin (1.0%) solution for 6 min followed by 4-5 washings with autoclaved distilled water (Sanghera et al., 2009b). The seeds were soaked in autoclaved distilled water for 6 hours and then sown in jam jars containing MS (Murashige and Skoog, 1962) medium supplemented with 8 g/l agar for germination at 28 ± 2°C, the seeds jars were kept in the dark. All sterilization work was performed in a laminar airflow cabinet. From germinating seeds, shoot apex with apical meristems were excised using sterilized scalpel blades and used in transformation studies

#### **Agrobacterium Strain and Plasmid**

Agrobacterium strain GV3101 having the plasmid pPZP200loxnosnptII::35SdeOmega Cry 1Ac vector was grown in Luria-Bertani liquid medium (tryptone 5 g l<sup>-1</sup>, NaCl 10 g l<sup>-1</sup>, yeast extract 5 g l<sup>-1</sup>) supplemented with 50 mg l<sup>-1</sup> kanamycin and 10 mg l<sup>-1</sup> rifampicin at 28°C for 24 h. The T-DNA of pPZP200 contains Cry1Ac ver-II and a kanamycin resistance gene (nptII) with intron in the N-terminal region and a GUS gene with CaMV 35S promoter and NOS terminator. The bacteria were resuspended in liquid MSB1 medium and the standard OD600 was adjusted to 0.3-0.5 (OD600=1 is equivalent to 1×10<sup>11</sup> cells per liter).

#### **Agrobacterium mediated Transformation conditions**

Shoot tips excised from 5 day old seedlings were injured gently on apical meristematic region with the help of sharp blade were inoculated with the Agrobacterium suspension and subsequently blotted dry with sterile filter papers. To optimize Agrobacterium mediated genetic transformation conditions different experiments were conducted based on concentration of bacterium (1000 µl, 500 µl and 100 µl broth in 25 ml of liquid MS medium), time for infection (10 min, 30 min and 60 min), infection method (spot and dip), co-cultivation period (24, 48 and 72 hours) and selection of putative transformants on selection medium (½ MS + 50 mg/l Kanamycin). Lethal dose of the antibiotic was determined by culturing the untreated (control) shoot apices on shoot induction medium containing different concentrations (0, 10, 25, 50, 75, 100, 125, 150 mg/l) of kanamycin. Based on this study 50 mg/l kanamycin was used for selecting the transformants on regeneration medium supplemented with cefotaxime (250 ppm) to avoid bacterial contamination. Regenerated shoots were allowed to grow on selection medium for 90 days (25+25+25+15).

#### **GUS Assay**

Histochemical GUS assay was carried out for shoot tips explants after 3 days of co-cultivation in Agrobacterium experiment using GUS assay solution consisting of sodium phosphate buffer and X-gluc (5 bromo, 4 Chloro, 3-indolylglucuronic acid) as substrate using the method described by Jefferson (1987).

#### **PCR Analysis**

Plants regenerated from Agrobacterium co-cultivated shoot apices survived up to 90 days were analysed for the presence of Cry1Ac. Genomic DNA was isolated from tender tissue of putative transformed plants using mini-prep DNA extraction method. Quantification of genomic DNA was done by

electrophoresing the DNA on 0.8% agarose gel with EtBr staining. PCR analysis was carried out using Cry1Ac specific forward primer 5'TGG AGA ACG CAT TGA AAC CG3' and reverse primer 5'TGT TGC TGA ATC CGG AAC GG3'. In vitro amplification using polymerase chain reaction (PCR) was performed in a PTC-100 thermal cycler (MJ Research, MA). The PCR mix for one reaction of 20 µl volume was placed in 96 wells thermal cycler. Amplification was performed using Initial denaturation at 94°C for 1 minute, annealing at 55°C for 1.30 min and extension at 72°C for 2 min and whole process of denaturation to extension was repeated 35 times. The PCR products were loaded on 1% high resolution superfine agarose (Promega) prepared with 0.5X TBE buffer (45 mM Tris base, 45 mM boric acid, 1 mM EDTA). The PCR amplification products were resolved by running the gel. Bands were visualized under UV light and photographed using UVP gel documentation system

#### **Statistical Analysis**

The data recorded were analyzed according to completely randomized design (simple and factorial) analysis (Snedecor and Cochran, 1967) using statistical software CPCS-1 package developed by Cheema and Singh (1990). The data of percent were converted to arc sine value for the analysis of variance (ANOVA) where required. The significance of variation among the treatments were observed by applying 'F' test and critical differences (CD) at 5 percent level of significance were calculated and used to compare the means of treatments and interpretations were made accordingly.

#### **Results and Discussion**

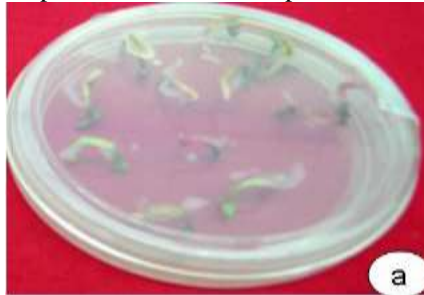
Though cotton has been transformed by Agrobacterium and plants have been regenerated (Umbeck et al., 1987, Firoozabady et al., 1987 and Perlak et al., 1990), success has been limited to Coker varieties that are highly regenerable. Commercially important cultivars have proven very difficult to regenerate under tissue culture conditions. In the present study, an attempt has been made to develop a method for transformation of shoot tip explants of LD 694 (diploid) and LH 2076 (tetraploid) cotton cultivars by co-cultivating with *A. tumefaciens* strain GV3101 carrying plasmid pPZP200 [CaMV 35S promoter, GUS reporter gene and nptII selectable marker]. To optimize Agrobacterium mediated genetic transformation conditions different experiments were conducted based on concentration of bacterium, time for infection, infection method and co-cultivation period for agroinfection, selection of putative transformants on selection medium and finally their molecular characterization using PCR analysis. The results obtained on aforementioned aspects are discussed in subsequent sections.

#### **Effect of Agrobacterium concentration**

In this experiment, excised shoot tips were injured in apical meristematic region prior to infection with Agrobacterium. The wounding may aid in the production of signal phenolics (Stachel et al., 1985) and enhance the accessibility of putative cell wall binding factors (Lippencott and Lippencott, 1969) to the bacterium. The exposure of shoot tip to undiluted (control) broth of Agrobacterium strain GV3101 containing Cry1Ac-II (OD600 = 0.5-0.6) resulted in overgrowth of the bacterium and severe necrosis of explants (Fig 1a).

So the broth was diluted in three concentrations viz. 1000, 500 and 100 µl/25ml and data were recorded on per cent explant infection and survival of infected explants. In first experiment, a total of 38 and 36 shoot tips of LD 694 and LH 2076, respectively were excised from 5-day old in vitro raised

seedlings and were gently injured in apical meristematic region and infected with 1000  $\mu$ l over night grown bacterial broth diluted in 25 ml of liquid  $\frac{1}{2}$  MS media. After infection with *Agrobacterium*, the shoot tips were cultured on half strength MS medium to allow the adhering *Agrobacterium* to transform wounded shoot tips. Two days after co-cultivation, overgrowth of *Agrobacterium* was observed all around the co-cultivated shoot tips (Fig 1b) resulting in 94.73 and 100.00 per cent infection in LD 694 and LH 2076, respectively. The overgrowth of *Agrobacterium* masked the growth of shoot tips and subsequently affected the regeneration of bacterium infected shoot tips. As a result, only 47.22 and 55.55 per cent of the infected shoot tips survived and developed into shoots (Table 1).



**Fig 1a Agrobacterium infection by dip method**



**Fig 1b Spotted method**

In other two experiments, bacterial broth was diluted using 500 and 100  $\mu$ l in 25 ml of liquid  $\frac{1}{2}$  MS media and used for co-cultivation of shoot tips excised from 5-day old in vitro grown seedlings. In these experiments, excised shoot tips from both the varieties were gently injured in apical meristematic region and co-cultured with *Agrobacterium* strain GV3101 (plasmid pPZP200). It is believed that wounding the shoot tip allows *Agrobacterium* to efficiently infect deep within the meristematic tissue. Gould and Magallanes (1998) also bisected the shoot from apex to base and removed excess tissue from stem base before inoculation with *Agrobacterium*. Lateral section of cotton shoot was removed to expose meristem in the apical region so that *Agrobacterium* might infect the apical meristem. The infected shoot tips were transferred to half strength MS medium and two days after co-cultivation on half strength MS medium, no over growth of *Agrobacterium* was observed and bacterial growth was restricted only to the explant (Fig 1c).



**Fig 1c Growth of Agrobacterium after 3 days in dip culture**

The survival percentage of shoot tips was high (70.00 - 72.22) in LD 694 and (68.00- 77.77) in LH 2076 as compared to survival of shoot tips in previous experiment described above (where 1000  $\mu$ l bacterial broth diluted in 25 ml liquid  $\frac{1}{2}$  MS media was used for infection of shoot tips with *Agrobacterium*). The reduced concentration of bacterial broth has been used in the earlier study by Umbeck et al., (1987) where 100  $\mu$ l of over night culture of *Agrobacterium* was used for infection of hypocotyl sections of *G. hirsutum* L. cv. Coker 310. Likewise, Firoozabady et al., (1987) placed cotyledonary pieces of plant tissues after agroinfection on sterile filter paper on callus initiation medium. In this study, bacterial concentration of 500  $\mu$ l was found ideal as it exhibited high percentage of explant infection (83.33 - 89.28 %) coupled with good survival (68.00 - 70.00 %) of infected explants without overgrowth of bacterium in both the varieties (Table 1).

#### Effect of inoculation method

In this experiment, excised shoot tips of LD 694 and LH 2076 from 5-day old seedlings were injured in apical meristematic region prior to infection with bacterium. The explants of both the varieties were inoculated with *Agrobacterium* strain GV3101 diluted to a concentration of 500  $\mu$ l /25ml  $\frac{1}{2}$  MS by using dip and spot methods for a period of 10 min and co-cultivated for 48 and 72 hrs on  $\frac{1}{2}$  MS medium. The infected shoot tips were transferred to half strength MS + 250mg/l cefotaxime medium after co-cultivation on half strength MS medium. Data recorded on percent explant infection and survival based on inoculation method showed that spotted inoculation exhibited higher infection rate (83.92% and 85.41%) than dipping method (69.68% and 73.80%) in both LD 694 and LH 2076 varieties (Table 2). The over growth of *Agrobacterium* was observed on explants in spotted method whereas bacterial growth was restricted only to the explants in dipped method. The spotted explants had shown necrosis with excessive bacterial growth (Fig 1d) and it was difficult to control at later stages that led to poor survival of 41.45 and 47.62 per cent of infected explants in LD 694 and LH 2076 varieties, respectively.



**Fig 1d Spotted culture**

However dipped explants did not show any necrosis but took longer time for the appearance of bacterial growth and showed comparatively high survival 59.64% and 66.55% of infected explants in both the genotypes thereby suggesting dip method as better choice for *Agrobacterium* mediated genetic transformation.

#### Effect of co-cultivation period

The shoot tip explants of varieties LD 694 and LH 2076 were inoculated with *Agrobacterium* strain GV3101 (diluted to a concentration of 500  $\mu$ l /25ml  $\frac{1}{2}$  MS) using dip and spot methods and co-cultivated for 48 and 72 hrs on  $\frac{1}{2}$  MS medium at 28°C. The infected shoot tips were transferred to half strength MS + 250mg/l cefotaxime medium after co-cultivation. Data recorded on per cent explant infection showed that co-



cultivation of explants for 72 hrs has higher infection rate (80.00%, 92.85%) for both the inoculation methods in variety LD 694 as compared to co-cultivation period of 48 hrs that has per cent infection of 59.37 and 75.00 in dip and spotted method, respectively. Similarly, in variety LH 2076 co-cultivation period of 72hrs exhibited higher percent explant infection than 48hrs in both the inoculation methods (Table 2), suggesting 72hrs co-cultivation period as an important factor for high agroinfection in cotton transformation using *Agrobacterium* method.

Following two and three days of co-cultivation on half strength MS medium, explants were cultured on regeneration medium i.e. half strength MS supplemented with carbenicillin 100 mg/l and cefotaxime 250 mg/l. The presence of carbenicillin and cefotaxime regulated limited growth of *Agrobacterium* in culture. Cefotaxime (50-100 µg/ml) and carbenicillin (400-500 µg/ml) has also been reported to be used to control the over growth of *Agrobacterium* by Umbeck et al (1987). Likewise, Leelavathi et al., (2004) cultured cotyledonary embryos after treatment with *Agrobacterium* on medium containing 250 mg/l cefotaxime. Similarly, Firoozabady et al., (1987) transferred hypocotyl sections to medium containing 500 mg/l carbenicillin after two days of co-cultivation with *Agrobacterium*.

The survival of infected shoot tips was high (66.66%) and (72.00%) when inoculated with dip method and co-cultivated for 72 hrs in both LD 694 and LH 2076 varieties compared to survival of shoot tips co-cultivated for 48 hrs having survival of 52.63% and 61.11% (Table 2). However, in spotted inoculation method co-cultivation period of 48 hrs appeared better as it recorded slightly higher (44.44 and 52.94) percentage of shoot survival than 72 hrs co-cultivation (38.46 and 42.30) in varieties LD 694 and LH 2076. In this study regeneration efficiency of transformed shoot tips was observed to be low because infection of shoot tips with *Agrobacterium* and injury made in the meristematic region of shoot tips along with callus induction might be causing suppression of growth of shoot tips and ultimately resulting in lower regeneration potential. The over growth of *Agrobacterium* observed in spotted method was found responsible for poor survival of infected explants. The survival percentage of shoot tips was high when co-cultivated for 72 hrs compared to survival of shoot tips co-cultivated for 48 hrs using dip method in this experiment and was used for infection of shoot tips with *Agrobacterium* in further experiment.

#### **Antibiotic selection of shoot tips infected with *Agrobacterium***

The regenerated shoots of both the genotypes were allowed to grow on half strength MS medium supplemented with 250 mg/l cefotaxime and 100 mg/l carbenicillin for three subcultures to achieve proliferation. Plantlets growing on regeneration medium were transferred to selection medium i.e. half strength MS supplemented with kanamycin @ 50 mg/l for identification of putative transgenic tissues/ plantlets. The choice of antibiotic used for selection is based on the chimeric GUS gene construct in pPZP200 plasmid that carries nptII selectable marker. Therefore, the selection of putative transformed shoots was carried out on half strength MS containing kanamycin. The concentration of antibiotic used for selection @ 50 mg/l was based on the antibiotic sensitivity test of shoot tips of LD 694 and LH 2076 (Table 3).

In the present study a total of 44 shoots of LD 694 were regenerated from shoot tips infected with *Agrobacterium*. Out of 44 regenerated shoots, 35 shoots were transferred to selection

medium i.e. half strength MS supplemented with 50 mg/l kanamycin (Table 4). After 15 days on selection medium, leaves of 35 regenerated shoots started to turn pale and withered (Fig 1e), whereas, 5 shoots on half strength MS without kanamycin remained green. After first selection of 30 days, 19 shoots out of 35 shoots of LD 694 survived, remaining turned dark brown and got eliminated (Table 4).



**Fig 1e** Shoot tips explants on selection medium containing cefotaxime (250mg/l) and Kanamycin 50 mg/l)



**Fig 1f**

Surviving shoots were further screened on selection medium i.e. half strength MS supplemented with 50 mg/l kanamycin. New leaves emerged from the axillary buds of surviving shoots. After second selection, 8 shoots out of 19 shoots of LD 694 survived which were further allowed to grow on media containing kanamycin for 20 days.

Only four shoots of LD 694 survived after third selection (Fig 1f). Similarly, for LH 2076, a total of 50 shoots were regenerated from shoot tips inoculated with *Agrobacterium*, out of which 37 shoots were transferred to selection medium supplemented with kanamycin and 10 shoots were cultured on half strength MS medium (Table 4). Fig 1g shows transformed regenerated shoots of LH 2076 growing on half strength MS medium without kanamycin. After first selection cycle of 30 days, 18 shoots out of 37 shoots of LH 2076 survived on medium containing kanamycin and remaining did not survive and got eliminated.



**Fig 1g** Shoots growing on regeneration medium

After second round of selection, 8 out of 18 shoots survived, which were further screened on selection medium containing kanamycin for another 20 days, and only 5 shoots of LH 2076

survived (Fig 1h). The survival percentage was 11.42 and 13.51 for LD 694 and LH 2076, respectively.



**Fig 1h**

These results demonstrate that transformed plants are able to survive on kanamycin supplemented media for 80 days, indicating that they might carry the transgene. Kumar and Rathore (2001) used kanamycin (conc. 50 mg/l) as selective agent to identify the hypocotyl segments transformed by LBA 4404 harboring pBINmGFPS-ER. Likewise, Chaudhary et al., (2003) used 50 mg/l kanamycin for selection of transformed somatic embryos of *G. hirsutum* cv. Coker 310 FR.

#### **Molecular characterization of putative transgenic cotton tissues/ plantlets**

Three days after co-cultivation the histochemical GUS assay was done that showed GUS expression in shoot tips varied from 5.55 to 12.50% (Table 5). The GUS expression in shoot tips and control are shown in Fig 2a.



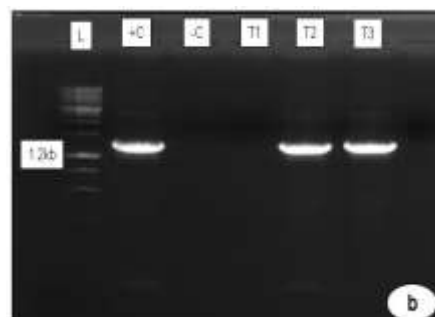
**Fig 2a Shoot tips showing GUS expression (blue tips) along with control**

The variable transient GUS expression (0-30 per cent) in different explants using *Agrobacterium* transformation have been reported by Kumar et al., (2007) who found highest 30.0% GUS expression in calli followed by 18.5% by cotyledons, 16.6% in shoot tips and 12.5% in hypocotyls explants that was comparable to reported earlier (Rajasekaran et al., 1996 and Zapata et al., 1999).

Further, survival of shoots/plants on selection medium (kanamycin) is not a proof of transformation. Yet, it is thought to narrow down the number of individuals to be tested for the presence of transgene. Differential exposure of the in vitro cultures to the selective agent, residual resistance of the plant tissue to the selective agent and the level applied might affect the outcome. Polymerase chain reaction was used in the present investigation to detect presence of Cry1Ac in putative transgenic tissues/ plantlets. Genomic DNA was isolated from tender leaves and/or stem tissues of 4 putative transgenic cotton shoots from LD 694 and 5 from LH 2076 selected after 3rd selection in particle gun bombarded experiment using small scale preparation. The DNA was quantified using agarose gel run and sufficient (25-150 ng) quantity of DNA could be isolated from 2 shoots in LD 694 and 3 in LH 2076 only. Plasmid carrying Cry1Ac gene cassette was used as a positive control and non-

transformed cotton leaf genomic DNA as negative control. Upon the completion of PCR with the specific primers, products were analysed on one per cent agarose gel electrophoresis. Fragment corresponding to Cry1Ac gene in positive control was observed and amplification was detected in the putative transgenic plantlets suggesting the presence of Cry1Ac with 1.2 KB product size (falling between 1KB and 2KB bands of 10KB DNA ladder), however no amplification was detected in non transgenic plant DNA used as control during the experiment (Fig 2b) confirming that these putative transgenic tissues carry Cry1Ac gene.

The low frequency of PCR positive plants among the selected shoots from tissue culture selection regimes is dependent on several factors such as tissue type, size of explant, chemical properties and concentration of selection agent and time of application (Bowen et al., 1998). There was very poor correlation between the plants, which survived on selection medium and their being positive for the presence of the transgene. Optimization of protocol in recalcitrant varieties like cotton enables improvement of tissue culture methods to induce efficient regeneration and transformation in a genotype independent manner and production of large number of transgenics.



**Fig 2b PCR analysis confirming the presence of Cry 1Ac in putative transformants**

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**Table 1: Effects of *Agrobacterium* concentration on infection and survival of shoot tip explants of cotton exposed to GV3101 strain containing *CryIAC* verII gene**

Varieties	<i>Agrobacterium</i> conc in $\frac{1}{2}$ MS ( $\mu$ l/25ml)	No. of Explants Inoculated*	No. of Explants Infected	Explants Infected (%)	No. of Explants Survived	Survival of Infected Explants (%)	Remarks
LD 694	1000 $\mu$ l	38	36	94.73	17	47.22	Overgrowth of bacterium
	500 $\mu$ l	24	20	83.33	14	70.00	No overgrowth
	100 $\mu$ l	36	18	50.00	13	72.22	-do-
	Undiluted (control)	28	28	100.00	0	0.00	Severe necrosis
LH 2076	1000 $\mu$ l	36	36	100.00	20	55.55	Overgrowth of bacterium
	500 $\mu$ l	28	25	89.28	17	68.00	No overgrowth
	100 $\mu$ l	32	18	56.25	14	77.77	-do-
	Undiluted (Control)	24	24	100	0	0.00	Severe necrosis
CD 0.05				6.94		7.02	

**Table 2: Effects of inoculation method and co-cultivation period on agroinfection and survival of shoot tip explants in cotton on half MS + 250 mg/l cefotaxime + carbenicillin 100 mg/l medium after 3 weeks**

Variety	Inoculation Method	Co-cultivation Period (hrs)	No. of Explants Inoculated*	No. of Explants Infected	No. of Explants Survived	Survival of Infected Explants (%)
LD694	Dipped	48	32	19 (59.37)	10	52.63
		72	30	24 (80.00)	16	66.66
	Mean			69.68		59.64
	Spotted	48	24	18 (75.00)	8	44.44
		72	28	26 (92.85)	10	38.46
	Mean			83.92		41.45
LH2076	Dipped	48	28	18 (64.28)	11	61.11
		72	30	25 (83.33)	18	72.00
	Mean			73.80		66.55
	Spotted	48	24	17 (70.83)	9	52.94
		72	26	26 (100.0)	11	42.30
	Mean			85.41		47.62
CD at 0.05				5.32		3.65

No. based on average of two repeats; Figures in the parentheses are % values

**Table 3: Effect of antibiotics on *in vitro* cultured shoot apices of cotton cultivars LD 694 and LH2076**

Varieties/ Antibiotic media	LD 694			LH 2076		
	Half MS	No. Explants Cultured*	No. of Explants Survived after 3 Weeks	Survival (%)	No. Explants Cultured*	No. of Explants Survived after 3 Weeks
Kanamycin (mg/l)						
0	24	22	83.33	16	14	87.50
10	28	14	50.00	20	11	55.00
25	22	5	22.72	20	4	20.00
50	24	3	12.50	20	2	10.00
75	24	0	0.00	18	0	0.00
100	26	0	0.00	18	0	0.00
125	28	0	0.00	24	0	0.00

No. based on average of two repeats

**Table 4: Selection of *Agrobacterium* transformed shoot tips of cotton on half MS + 250 mg/l cefotaxime + 50 mg/l kanamycin medium**

Varieties	No. of Shoot Tips Inoculated	No. of Shoot Tips Infected	No. of Shoot Tips Survived and Regenerated on ½ MS +250mg/l Cf medium after 1week	No. of Regenerated Shoots put on Selection Medium	No. of Shoots Survived during Selection			Survival after 3 <sup>rd</sup> selection (%)
					After 1 <sup>st</sup> selection	After 2 <sup>nd</sup> selection	After 3 <sup>rd</sup> selection	
LD 694	28	24 (85.71)	15 (62.50)	12	9	5	2	16.66
	24	19 (79.16)	11 (57.89)	8	4	-	-	0.00
	36	28 (77.77)	18 (64.28)	15	6	3	2	13.33
Total	88	71 (80.68)	44 (61.97)	35	19	8	4	
Mean								11.42
LH 2076	36	27 (75.00)	19 (70.37)	15	8	3	2	13.33
	28	22 (78.57)	14 (63.63)	10	5	2	1	10.00
	32	26 (81.25)	17 (65.38)	12	6	3	2	16.6
Total	96	75 (78.12)	50 (66.66)	37	18	8	5	
Mean								13.51
CD at 0.05		6.58	5.85					2.52

Values in the parenthesis are in per cent

**Table 5:  $\beta$ -glucuronidase expression in different shoot tip explants of cotton after inoculation with *Agrobacterium tumefaciens* strain GV3101**

Variety	No. of Explants Treated	No. of Explants found GUS +ve	Explants Showing GUS Expression (%)	Mean
LD 694	24	3	12.50	11.66
	20	2	10.00	
	16	2	12.50	
LH 2076	16	2	12.50	9.04
	22	2	9.09	
	18	1	5.55	