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Conditions for efficient transformation of tomato (*Lycopersicon esculentum* M.) cultivars with cry1a(c) gene of *Bacillus thuringiensis*

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Abstract
The efficacy of tomato cultivars Hisar Lalit and Hisar Arun to regenerate on different media combinations and their frequency of transformation with cry 1A(c) gene of Bt was evaluated. Experiments were conducted to develop an efficient and reproducible plant regeneration system in two cultivars which are high yielding. conditions were standardized for various factors which affect transformation efficiency like, preculture of explants, inoculation time and co-cultivation duration, using a binary vector pCAMBIA 1301 harbouring *gus* as a reporter gene. *Agrobacterium*-mediated genetic transformation was carried out for the above mentioned tomato cultivars for introduction of *Bacillus thuringiensis* cry1A(c) gene. For this the binary vector pBin 19 was used which contained cry1A(c) gene and *npt II* as selectable marker (for kanamycin resistance) controlled by CaMV35S promoter. Both these binary vectors (pCAMBIA 1301 and pBin 19) were mobilized into *Agrobacterium tumefaciens* strain EHA 105. The explants were precultured for three days, treated with bacterial solution for 20 minutes and then co-cultivated for 48 hours. Then these explants were put under a selection pressure of 100 mg/l kanamycin. The control plants bleached while the transformants containing the cry1A(c) gene of Bt and kanamycin resistance gene were healthy, green and survived. This clearly shows that Bt gene along with selectable marker kanamycin have been integrated into the plant genome. The transformation efficiency was recorded and it was observed that cotyledons of cv. Hisar Arun showed 79 per cent while cv. Hisar Lalit showed 81 per cent transformation frequency. In case of hypocotyl explants there was a reduction in transformation efficiency viz. 66 per cent in case of cv. Hisar Arun and 75 per cent in cv. Hisar Lalit.

Keywords: Transformation, *Agrobacterium*, selectable marker, inoculation, kanamycin, *gus* gene

Introduction
Agrobacterium has opened a plethora of opportunities to transfer foreign DNA sequences of desired interest into various plant species. Tomato is the best suited vegetable for biotechnological manipulations due to its enormous wealth of genetic background and the availability of a comprehensive collection of germplasm. Tomato is extensively damaged by the Lepidopteron insect *Helicoverpa armigera* also called tomato fruit borer. Its attack is confined to the stem – end of the fruit. The degree of incidence depends upon the fruit number, earliness, fruit size and plant density. It causes heavy reduction in yield. Explant transformation using *A. tumefaciens* has been reported to be best applicable to all or most of the tomato genotypes. The relative simplicity of this technique and the genetic stability of the transformants obtained, particularly with respect to ploidy level, make this technique preferable when aiming at the transfer of desirable genes into tomato. A prototype vector pCGN1547 was found to be consistently stable in *A. tumefaciens* strain LBA4404 and proved to be an efficient donor of T-DNA in tomato transformation experiments (Mc Bride and Summerfelt, 1990) [8]. *Agrobacterium*-mediated transformation in tomato was first reported by McCormick *et al*. (1991) [10]. Other procedures for *L. esculentum* transformation include those of Fillatti *et al*. (1987) [4] and Bird *et al*. (1988) [2]. Looking into the possibility and ease of extending beneficial gene pool to tomato through genetic engineering, a pragmatic approach has been put forward to generate insect resistant tomato. The present investigation was carried out to introduce cry1A(c) gene of *Bt* in tomato cultivars Hisar Arun and Hisar Lalit after optimizing the conditions for *Agrobacterium* – mediated transformation using *gus* as reporter gene.

Material and Methods
Plant material
Two tomato cultivars, Hisar Lalit and Hisar Arun were used in the present investigation. Hisar Lalit (NT-8), a hybrid between Bangalore and HS 101, is a highly root knot nematode

resistant, high yielding, semi-determinate variety. Hisar Arun (Sel-7), is a selection from PED × K1 and is very early, high yielding, determinate, dwarf with concentrated fruiting type of variety.

Plasmids and Bacterial strains

Plasmid pCAMBIA 1301 mobilized into *Agrobacterium tumefaciens* strain EHA 105 was used for the optimization experiments, this strain was provided by CAMBIA, Australia. For plant transformation, the binary vector pBin 19 mobilized into *A. tumefaciens* strain EHA 105 (Hofgen and Willmitzer, 1998) [5]. This binary vector pBin 19 harbors the *Bt* gene cry1A(c) that codes for an insecticidal crystal protein, against *Helicoverpa armigera* (Table 1)

Nature of Explants

15 to 20 days old seedlings, grown in vitro were excised in the laminar hood to extract the explants. The most frequently used explants were hypocotyls and cotyledons. Leaves were also used they were cut in the form of discs.

Response of explants of tomato cultivars Hisar Lalit and Hisar Arun to Kanamycin

The sensitivity and response of the cotyledons, hypocotyls and leaf explants, of both the tomato cultivars, to Kanamycin was studied by culturing them on their regeneration media containing various concentrations of Kanamycin (0, 25, 50, 75, 100mg/l). With this the optimum concentration of Kanamycin for survival and shoot regeneration from the explants was determined.

Optimization using *gus* as reporter gene

Agrobacterium tumefaciens strain EHA 105, harboring, pCAMBIA 1301 vector was used to optimize the factors which influence the transformation experiments, discussed in later sections. This binary vector pCAMBIA 1301 contains *gus* which acts as reporter gene and is driven by CaMV35S promoter.

The explants were pre cultured for 0,1,2,3 and 4 days on the pre-culture media containing zeatin (1.0 mg/l) (Table 2). The infection times of 5, 7, 10, 20 minutes on different explants were studied separately with 20 folds diluted *Agrobacterium* culture were assayed after co-cultivation on co-cultivation media. The effect of co-cultivation duration on plant transformation, using histochemical GUS assay for 24, 48 and 72 hours were studied.

Histochemical determination of gus activity in plant tissues was conducted according to Jefferson *et al*. (1987) [6] protocol using x-gluc as substrate and transient expression of gus gene was detected. The different explants, after co-cultivation were incubated in the GUS assay solution at 37ºC overnight and scored for evidence and extent of transient *gus* activity. The plant tissues were scored as gus positive (gus+) if any blue spot was observed on the tissue or as gus negative (gus–) if no blue stain was detected on the explants.

Transformation of tomato cultivars with insect resistant *Bt* gene, cry1A(c)

Using the Bt gene cry1A(c) in the vector pBin19 driven by promoter CaMV35S, mobilized into *A. tumefaciens* strain EHA 105 the tomato explants were transformed with the insecticidal crystal protein. Following the conditions optimized the tomato explants were pre-cultured for three days and dipped into the overnight grown, 20 fold diluted, *Agrobacterium* and incubated for 20 minutes with occasional swirling. The explants were blotted dry on the sterilized Whatman No.1 filter paper and placed (with cotyledons and leaves placed upside down) on the co-cultivation medium. The explants were co-cultivated for 48 hours in a growth room at 26ºC and 16 L/8 D photoperiod. Around 75-100 explants were co-cultivated in each petri-plate.

After 48 hours of co-cultivation the explants were transferred to the selection medium which had the appropriate concentration of Kanamycin as optimized and 500 mg/l cefotaxime. The culture conditions were 26ºC and 16 L/ 8D photoperiod. The green bumps or shoot bud formation was noted. After every 15-20 days the explants were sub-cultured on the selection medium and monitored for further shoot regeneration.

Results and Discussions

Highly efficient conditions for successful *Agrobacterium* mediated transformation has been developed for transformation of tomato cultivars with cry1A(c) gene. The optimized plant regeneration protocol (Mishra *et al*., 2007) [13] was used for tomato transformation experiments.

Response of explants of tomato cultivars to various concentrations of kanamycin

Kanamycin was found to have inhibitory effect on shoot regeneration of tomato cultivars. The response of explants to various concentrations of Kanamycin was evaluated by culturing the cotyledon and hypocotyls eplants of tomato on their regeneration medium with varying levels of kanamycin (0-100 mg/l). It was observed that at 0.00 mg/l kanamycin both the explants showed 100 per cent survival but as the concentration was increased the survival rate went on decreasing and at 100 mg/l Kanamycin concentration all the cotyledon and the hypocotyls explants bleached completely and no shoot regeneration of shoots observed (table 3). Therefore, 100 mg/l kanamycin was used in the selection medium in transformation experiments to introduce cry1A(c) gene of Bt. This inhibitory effect of Kanamycin on regeneration response of explants has also been reported by Bhatnagar *et al*., 2004[1]. This level of Kn concentration for transformed tomato selection has also been recommended by Mandaokar *et al*. (2000) [7] and McCormick (1991) [10]

Optimization of conditions for gene transfer using *gus* as a reporter gene

A. tumefaciens strain EHA 105 carrying the vector pCAMBIA 1301 was grown overnight in LB broth (Table 2) and used after 20-fold dilution for treatment of seedling explants. This plasmid contained *gus* as reporter gene for the synthesis of β-glucuronidase and *hpt II* gene for hygromycin selection of the explants. After *Agrobacterium* treatment the cotyledon, hypocotyls and leaf explants of both cultivars were incubated on their co-cultivation media and then assayed for GUS expression to optimize the pre-culture condition; inoculation time and duration of co-cultivation conditions for gene transfer.

Pre-culture of explants

Cotyledon, hypocotyl and leaf explants of both the tomato cultivars, excised from 15-20 days old *in vitro* grown seedlings were used to observe the effect of pre-culture on transformation efficiency. Prior to treatment with *Agrobacterium* all the three explants were pre-cultured on their regeneration media for 0, 1, 2, 3 and 4 days. Maximum

transient GUS expression was noted from three days pre-culture of all the three explants of both cultivars.

The hypocotyls from cv. Hisar Lalit showed highest transient GUS expression of 59.15 per cent on three days pre-cultured explants while freshly cut hypocotyls showed the lowest response of only 12.5 per cent. In case of cv. Hisar Arun also the three days pre-cultured explants of the hypocotyls showed highest transient GUS expression (60.15%).

Three days pre-culture of cotyledons of cv. Hisar Lalit showed highest GUS expression (60.00%) and that of cv. Hisar Arun showed 41.15 per cent. Similarly leaves also showed higher per cent of transient GUS expression when pre-cultured for three days viz. cv. Hisar Lalit leaves showed 38.50 per cent and cv. Hisar Arun leaves showed 22.90 per cent response.

Therefore, three days pre-culture was used for gene transfer purposes in both these cultivars and the response of hypocotyls and cotyledons of both cultivars were at par in pre-culture conditions (Fig. 1). Preculture has also known to enhance regeneration in tree species like Malus (Schaart *et al*., 1995) [12], (De Bondt *et al*., 1994) [3].

Inoculation time

The cotyledons and hypocotyls of both the tomato cultivars were used to optimize the inoculation time of these explants with *Agrobacterium* during treatment phase of the transformation protocol. For this 5, 7, 10 and 20 minutes of inoculation time were used to score for transient GUS expression.

The cotyledons of cv. Hisar Lalit showed highest (55.00%) transient GUS expression while those of cv. Hisar Arun showed a maximum of 45.00 per cent of GUS expression. The highest response was obtained from 20 minutes of infection/inoculation/treatment time in both the cultivars. The hypocotyls also showed a high response of 47.50 per cent in case of cv. Hisar Lalit and 50 per cent in case of cv. Hisar Arun, for transient GUS expression. Again 20 minutes inoculation time proved best for the hypocotyls also.

Therefore, the inoculation time of 20 minutes was optimized for explant treatment with *Agrobacterium* strain during the transformation experiments (Fig.2). Ninety minutes of inoculation time has been optimized for *Morus indica* cv. K2 with *Agrobacterium* strain LBA 4404 (Bhatnagar *et al*., 2004) [1].

Co-cultivation duration

To study the effect of co-cultivation duration of cotyledons, hypocotyls and leaf explants excised from 15-20 days old *in vitro* grown seedlings were co-cultivated with *Agrobacterium* strain EHA 105 carrying pCAMBIA 1301 on their co-cultivation media (Table 2) for different time intervals. Co-cultivation periods of 24, 48, 72 and 96 hours were used to determine the optimum co-cultivation time that resulted in highest transformation efficiency.

Seventy-two hours showed a maximum transient GUS expression in all the three explants of the tomato cultivars Hisar Lalit and Hisar Arun (Fig. 3). Inspite of this, 48 hours was used for the transformation purposes as the results were at par with 72 hours. After 72 hours co-cultivation duration caused heavy growth of *Agrobacterium* that kills the explants. Similar observations have been made in tomato cv. Pusa Ruby (Mandaokar *et al*., 2000) [7] and tomato cv. VF 36 (McCormick, 1991) [10]. Whereas, three days co-cultivation has been recommended for *Brassica* spp. by Mukkhopadhyay *et al*. (1992) [11].

Confirmation of optimization experiments for gene transfer

Genetic transformation of cotyledon, hypocotyl and leaf explants of the tomato cultivars Hisar Lalit and Hisar Arun with the vector pCAMBIA 1301 was confirmed by carrying out assay for *Gus* gene product.

Histochemical GUS assay

Gus expressions in the explants were determined through x-gluc staining. Appearance of blue colour near the cut ends of the tissues is an indicative of gus activity. Those explants which showed blue were scored as gus+ while those that did not were scored gus- (Fig. 4A). Standardization using histochemical GUS assay found that three days pre-culture tremendously increased the transformation frequency of all explants. 20 minutes of inoculation time and two days co-cultivation has been found to successfully transfer and integrate the bacterial plasmid DNA with the plant genomic DNA. With any other parameter it became very difficult to recover the explants due to bacterial over growth.

***Agrobacterium*-mediated transfer of cry 1A(c) gene of *Bt* in tomato**

The cotyledons and hypocotyls of both the cultivars of tomato were excised and placed on their regeneration media for three days pre-culturing. These explants were then treated with overnight grown 20-fold diluted *Agrobacterium* strain EHA 105 (harboring vector pBin 19) (Table 1) for 20 minutes. The explants were then blotted dry and co-cultivated for 48 hours as was optimized. After 48 hours of co-cultivation, the explants were shifted to the selection medium (Table 2) containing 100 mg/l kanamycin and 500 mg/l cefotaxime. Only few plants which were actually transformed and had the cry1A(c) gene with kanamycin selectable marker integrated, survived and rest of all the plants bleached and died (Fig.4 B-E).

The transformation frequency was worked out on the basis of plants growing on selection medium having 100 mg/l kanamycin and it was found that the cotyledons showed 79 per cent transformation frequency in case of cv. Hisar Arun while it was lower in cv. Hisar Lalit (81%). The hypocotyls also showed lower transformation frequency (66%) in case of cv. Hisar Arun and was lowest in case of cv. Hisar Lalit (75%) (Table 4). The control plants were all bleached while the shoots from the transformed explants were green (Fig.4 E-G).

Conclusion

Based on the present observations, it can be concluded that cotyledons are more amenable to *Agrobacterium*-mediated transformation than hypocotyls explants for both the cultivars used in the present investigation. By this protocol further research can be conducted to integrate some other valuable genes to these cultivars for further improvement. Towards this objective, genes coding for inhibitors of insect proteases and vegetative insecticidal proteins (VIP) and some nematode resistant genes may be considered for introduction into these varieties of tomato in conjunction with cry1A(c).

Table 1: Plasmids and bacterial strains

Plasmid	Agrobacterium strain	Gene of interest	Promoter	Antibiotic resistance marker gene for	
				Bacterial selection	Plant selection.
pCAMBIA 1301	EHA 105	*gus*	CaMV35S	Kanamycin	Hygromycin
pBin 19	EHA 105	Cry1A(c)	CaMV35S	Kanamycin	Kanamycin

Table 2: Media used for *Agrobacterium*-mediated transformation

Medium	Culture media Supplements	Ph
Pre-culture medium	MS basal* + Zeatin (1.0 mg/l)	5.8
LB broth for growth of *Agrobacterium*	LB + Kanamycin 50 mg/l Acetosyringone 200 µM	7.5
Co-cultivation medium	MS basal* + Zeatin (1.0 mg/l)	5.2
Selection medium	MS basal* + Zeatin 1.0 mg/l + Kanamycin 100 mg/l + Cefotaxime 500 mg/l	5.8
Subculture medium	MS basal* + Zeatin 0.1 mg/l + Kanamycin 100 mg/l + Cefotaxime 300 mg/l	5.8
Rooting medium	MS basal* + Kanamycin 50 mg/l + Cefotaxime 300 mg/l	5.8

*MS basal includes all salts of Murashige and Skoog medium + 3.0% sucrose + 0.8% Agar.

Table 3: Response of different concentrations of Kanamycin on tomato explants

Conc. of Kanamycin (mg/l)	Survival of explants on selection (%)			
	Hisar Arun		Hisar Lalit	
	Cotyledons	Hypocotyls	Cotyledons	Hypocotyls
0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
25	85.00 ± 0.06	83.00 ± 0.07	84.67 ± 0.43	82.89 ± 0.57
50	83.64 ± 0.03	82.85 ± 0.05	80.00 ± 0.26	75.00 ± 0.16
75	50.00 ± 0.20	47.60 ± 0.17	48.85 ± 0.08	46.00 ± 0.06
100	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Table 4: Transformation frequency

Varieties	Percent transformed shoots regenerated from hypocotyls (Mean ± S.E.)	Percent transformed shoots regenerated from cotyledons (Mean ± S.E.)
Hisar Arun	66.00 ± 0.23	79.00 ± 0.11
Hisar Lalit	75.00 ± 0.09	81.00 ± 0.13

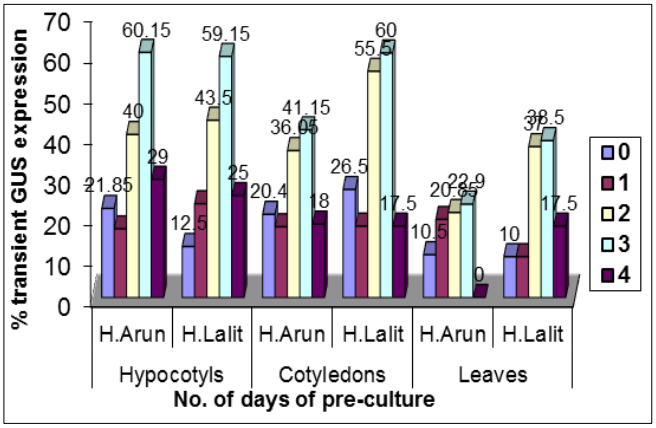

Fig 1: Effect of pre-culture of explants of Tomato cultivars on transient GUS expression

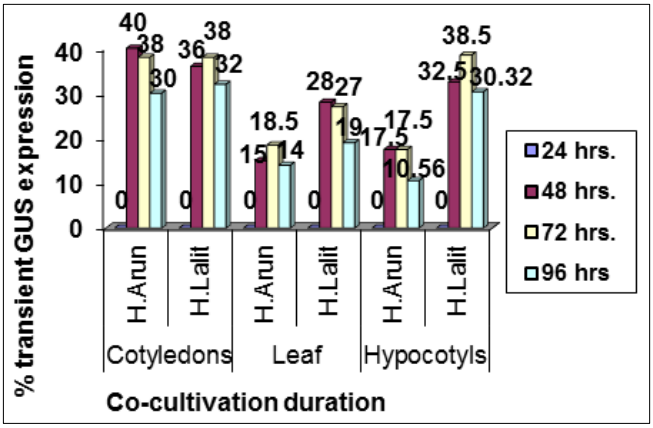

Fig 3: Effect of co-cultivation duration tomato explants on transient GUS expression

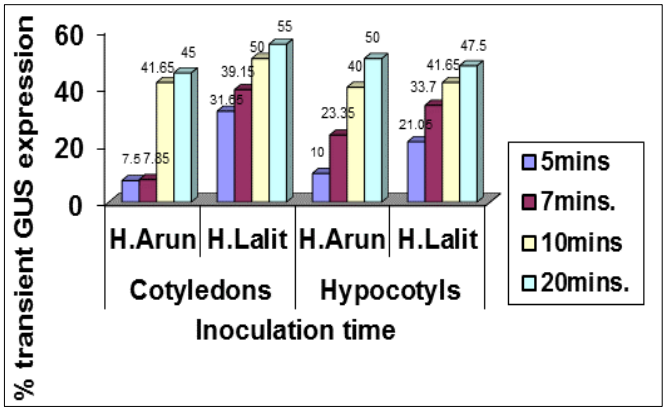

Fig 2: Effect of inoculation time of tomato explants on transient GUS expression

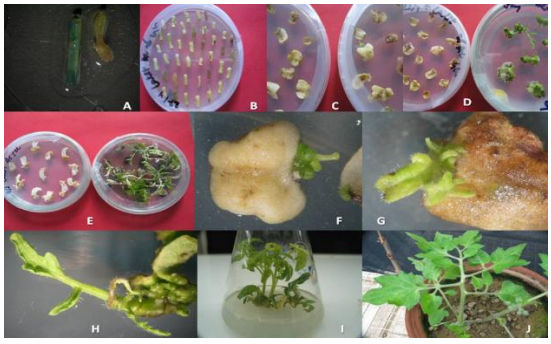

Fig 4: Transformation of tomato cultivars with cry 1 A(c) gene. (A) Trainsient gus expression in tomato; (B, C, D and E) Selection of transformants from non-transformants in selection medium; (F, G, H, I) Regeneration of transformed tomato plantlets; (J) Transgenic tomato successfully transplanted in soil in transgenic house.

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