Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env. Pharmacol. Life Sci., Vol 6 [2] January 2017: 74-82

Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD
Global Impact Factor 0.533
Universal Impact Factor 0.9804

ORIGINAL ARTICLE **OPEN ACCESS**

Enhancement of Transformation efficiency using *Agrobacterium* in *Gossipium hirsutum* (L.) through parameters optimization

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ABSTRACT
In cotton, transformation efficiency is subdued; despite production of fertile transgenic plants through Agrobacterium mediated transformation. Due of the lack of non-reproducing regeneration protocols and optimized parameters for transformation in cotton, the efficiency of cotton transformation remained poor. The present investigation was undertaken to optimize various parameters which enhances transformation frequency in PKV-Rajat cultivar in Gossipium hirsutum (L.) using Agrobacterium tumefaciens strain EHA 105 harboring cry1F gene linked to CaMV35S promoter. Observations were recorded as percent survival of explants on selection media (MS + Kanamycin @ 100mg/lit). The results revealed that high survival rate of explants could be attained through optimization of parameters such as colonization of explants with 0.6 bacterial OD and 100 µM acetosyringone concentration which can be used for transformation protocol to achieve high transformation rate in cotton plant.
Keywords: *Gossypium hirsutum, colonization, co-cultivation, acetosyringone, bacterial cell density*

Received 20.10.2016 Revised 15.12.2016 Accepted 02.01.2017

INTRODUCTION

Plant transformation has become an important experimental tool for the investigations of various aspects of plant biology such as physiology, genetics, molecular biology etc. Among the various techniques, *Agrobacterium tumefaciens* -mediated transformation is the preferred technique for the genetic modification of most of the plant species because of its simplicity of use, stable expression, ease of protocol and its propensity to introduce transgenes in low copy number [1, 2].

Agrobacterium-mediated transformation is the traditional method for obtaining transgenic plants through introgression at defined locus mostly in dicot plant species. Binns and Thomashaw in 1988 revealed that *A. tumefaciens* is capable of transferring a particular DNA segment Transfer (T)-DNA of the tumour-inducing (Ti) plasmid into the nucleus of infected cells where it is subsequently integrated into the host genome [3]. The virulent strain of *A. tumefaciens* contain a megaplasmid having size more than 200 kb that plays lead role in tumour induction and thus it named as Ti plasmid. Virulence genes (vir genes) are responsible for incorporation of Ti plasmid into the bacterial chromosome [4,5].

The Vir genes include phenolic compounds named acetosyringone and related acetophenones, benzaldehydes, benzonic acids, and cinnamic acid derivatives [6,7,8]. Acetosyringone, a phenolic compound secreted by wounded plant tissue and consider as a potent inducer of Agrobacterium vir genes8. Low concentration of acetosyringone leads the synergistic action of glucose and galactose for the induction of vir genes9 and such Induction of the vir gene by acetosyringone is enhanced by opines [10]. Several reports suggested that acetosyringone pre-induction of Agrobacterium in the co-cultivation medium can enhance the rate of transformation efficiency [11].

Cotton (*Gossypium hirsutum* L.) belongs to the family Malvaceae. The genus *Gossypium* comprises of 50 species. The most widely grown species *Gossypium hirsutum* L. are tetraploid with 2n=52. Early bud formation, lint strength and high yield are the major properties of *G. hirsutum.* Because of the available non-effective regeneration protocols, transformation efficiency of cotton cultivars has remained poor. Admezyk and Gore in 2004 have investigated the efficacy of transgenic cotton genotypes containing

cry1AC, cry1F and cry1AC stacked with cry1F against beet armyworms and fall army worm in the laboratory bioassays and small experimental field plots and observed that cotton containing cry1F was more toxic than cotton containing only cry1AC[12].

The two critical steps to be optimized for genetic transformation of plants are transfer of foreign DNA into the plant cells and regeneration of plants from transformed cells [13]. In many species, transformation efficiency is subdued because transformed cells unable to regenerate owing to several factors such as accessibility of cells for gene transfer [14]. A number of factors produced significant differences in T-DNA delivery; these included optical density, inoculation duration, co-cultivation time, acetosyringone concentration in co-cultivation medium and vacuum infiltration assisted inoculation.

As more researchers from diverse disciplines adopt transgenic approach for their investigations, there is a need for a comprehensive article explaining the critical factors to meet the challenges of cotton transformation.

MATERIALS AND METHOD

Selection of Plant Material: PKV-Rajat is the hirsutum hybrid which is moderately tolerant to bacterial blight, jassids and bollworm released by Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola, Maharashtra recommended for Vidarbha region used for the present investigation.

Preparation of Explants: Shoot tip and cotyledonary node were excised from 7-10 days old seedlings grown on ½MS, whereas, mature embryos were obtained from overnight soaked seeds carefully (figure 1). The explants were then precultured for 72 hrs on MS supplemented with 0.1mg/lit.

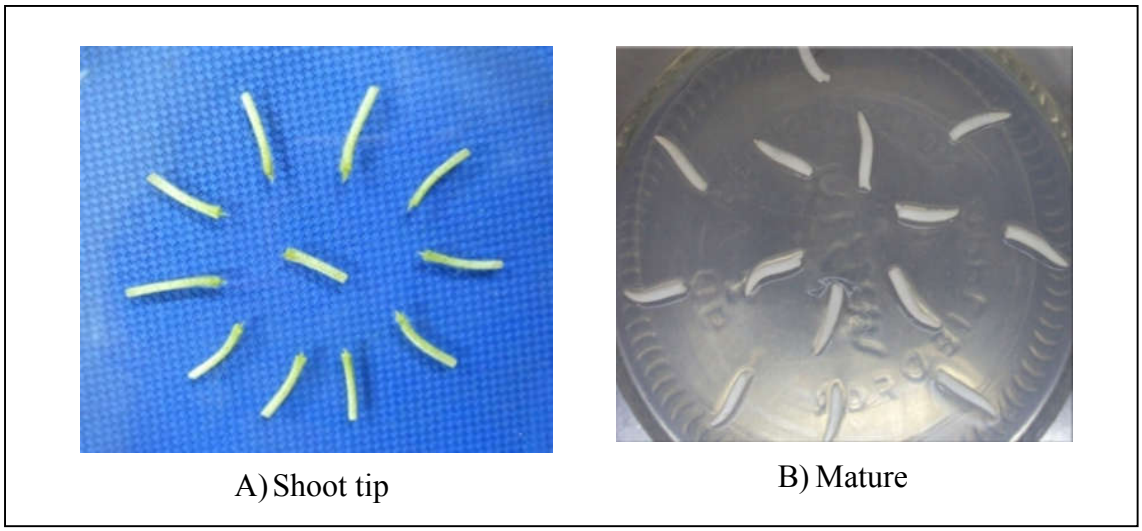

Figure 1: Explants taken during present investigation

Standardization of different parameters for *in vitro* transformation

Efficient transformation frequency could be achieved through the optimization of different parameters. Efforts have been taken for standardization of parameters such as optical density, acetosyringone concentration, colonization and co-cultivation time etc.

Effect of inclusion of acetosyringone during colonization

Virulence inducing agents such as acetosyringone is considered to be an important variable in *Agrobacterium* mediated transformation. To study the effect, variable concentrations of acetosyringone such as 100 µM, 200 µM and 400 µM were used in *Agrobacterium* suspension, whereas, culture lacking acetosyringone considered as control. The inoculum was prepared from overnight grown *Agrobacterium*. The culture was then centrifuged at 8000 rpm for 10 minutes and the pellet was suspended in ½MS broth according to OD required. Varying concentrations of acetosyringone (@ 0 µM, 100 µM, 200 µM and 400 µM was added to the suspended culture. Explants were immersed in *Agrobacterium* suspension for colonization and co-cultivated on preculture media in dark condition.

Bacterial cell density

Efficient T-DNA integration could be achieved through the infection with minimum bacterial population. To optimize bacterial cell density for transformation, experiment was conducted with all the three explants. The explants were co-cultivated with bacterial suspensions of three different OD values read at 600 nm (viz., 0.6, 0.8 and 1.0) keeping ½ strength MS broth as control. These explants were co-cultivated on MS with kinetin @ 0.1 mg/lit.

Colonization of explants

In order to standardize colonization duration, the *Agrobacterium* were grown in Yeast Extract Mannitol broth for 48 hrs with 150 rpm. The culture is then centrifuged at 8000 rpm and the pellet was suspended in ½ strength MS broth further explants were colonized with suspended culture for 15 min, 25 min and 35 min in dark. Explants without colonization served as control. After colonization, the explants were transferred to shoot induction media.

Co-cultivation of explants

For the optimization of co-cultivation duration, different durations were tested as 2, 3, 4 and 5 days. Explants transferred directly on preculture media were considered as control. At the end of the co-cultivation period, explants were transferred to a medium containing an antibiotic cefotaxime that will specifically inhibit bacterial growth but without selection agent kanamycin as in cotton T-DNA integration and transformation rate is too less. Therefore in the present study selection pressure was applied after shoot induction from transformed cell.

RESULTS AND DISCUSSION

Secretion of phenolic compounds through wounded tissue stimulates transformation. Acetosyringone, is known to increase virulence of *Agrobacterium*. Cotton shoot apical meristems do not release phenolic compounds or other signal molecules in sufficient quantities so as to elicit *vir* gene induction. With the inclusion of acetosyringone, it is likely that signal molecules are sufficiently released to elicit *vir* gene induction [15].

In the present study 100 µM, 200 µM and 400 µM of acetosyringone in a *Agrobacterium* culture medium were tried for the three explants (shoot tip, cotyledonary node and mature embryo) and observations were recorded for percent survival of shoots on kanamycin medium after four, eight and twelve weeks of infection. The results are presented in table 1 and figure1 indicates that the survival was higher upto 4 weeks on selection medium and reduced after 8 weeks in case of all the explants. Amongst the explants, mature embryo showed higher survival than shoot tip and cotyledonary node at 100µM acetosyringone concentration.

Table 1: Effect of various acetosyringone concentrations on transformation efficiency

Explants	Acetosyringone concentration	Percent survival on selection medium			Mean
		4 weeks	8 weeks	12 weeks	
Shoot Tip	Control	0.33	0.00	0.00	0.11
	100 µM	0.67	0.33	0.00	0.33
	200 µM	0.33	0.00	0.00	0.11
	400 µM	0.00	0.00	0.00	0.00
Mean		0.33	0.08	0.00	
Cotyledonary Node	Control	0.33	0.00	0.00	0.11
	100 µM	0.67	0.33	0.00	0.33
	200 µM	0.33	0.00	0.00	0.11
	400 µM	0.00	0.00	0.00	0.00
Mean		0.33	0.08	0.00	
Mature Embryo	Control	0.33	0.33	0.00	0.22
	100 µM	1.00	0.67	0.00	0.57
	200 µM	0.67	0.33	0.00	0.22
	400 µM	0.00	0.00	0.00	0.00
Mean		0.42	0.33	0.00	
Grand Mean		0.36	0.16	0.00	
SE (m) ± (A)		0.19	0.16	-	
CD (5 percent) (A)		0.55	0.45	-	
SE (m) ± (B)		0.16	0.13	-	
CD (5 percent) (B)		0.48	0.39	-	
SE (m) ± (AxB)		0.33	0.27	-	
CD (5 percent) (AxB)		0.96	0.79	-	

(figures in the parenthesis are transformed values)

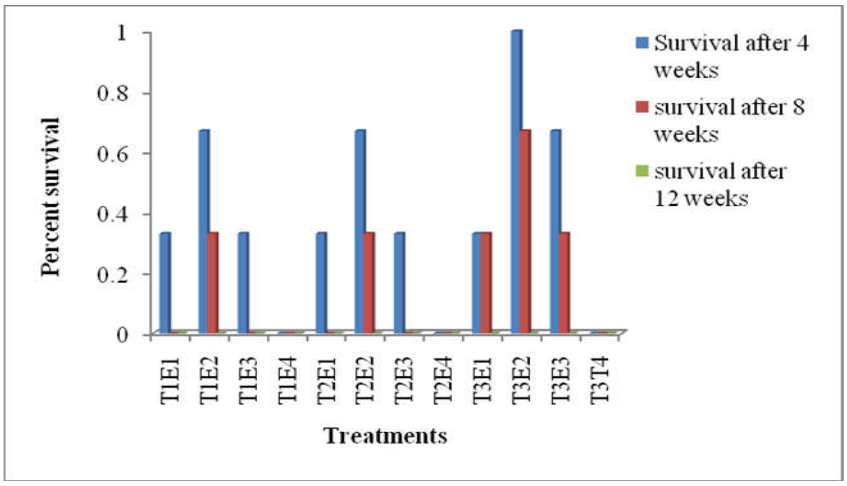

Fig.1 Effect of various acetosyringone concentrations on transformation efficiency

Data revealed that the effect of acetosyringone concentration with explants are significant for percent shoot survival after 4 weeks and 8 weeks on selection medium, and 100 µM acetosyringone was found best with all the three explants under study.

Kanthi (2006) had also reported 100 µM of acetosyringone as best treatment yielding maximum putative transformants (25.05 percent) from cotyledonary node explants of both Maruti and Asha genotypes of pigeonpea[16]. The results of present investigation are in agreement with the results obtained by Saharan et.al in 2004 in Indica rice cultivars HKR-46 and HKR-126. They had reported improvement in *Agrobacterium*-mediated transformation using 100µM concentration of acetosyringone in the *Agrobacterium* culture [17]. Droste *et al*. (2000) also used 100 µM of acetosyringone in order to increase the virulence of *Agrobacterium tumefaciens* in soybean [18]. The transformation efficiency of 15.62 percent was obtained in wheat when acetosyringone concentration of 100 µM was used [19].

Bacterial cell density

Optimum concentration of *Agrobacterium* is important because, though it may not really influence the frequency of transformation, it will surely influence the growth of explants after co-cultivation. Because higher cell density leads to excess growth of *Agrobacterium*, and the elimination of it from explants becomes difficult. The explants were co-cultivated with different concentration of bacterial suspension (O.D. measured at 600 nm) such as 0.6, 0.8 and 1.0, wherein the explants survived without yellowing and death.

Table 2: Effect of bacterial cell density on transformation efficiency

Explants	Bacterial Cell Density	Percent survival on selection medium			Mean
		4 weeks	8 weeks	12 weeks	
Shoot Tip	Control	0.00	0.00	0.00	0.00
	0.6	0.67	0.33	0.00	0.33
	0.8	0.33	0.00	0.00	0.11
	1.0	0.00	0.00	0.00	0.00
Mean		0.25	0.08	0.00	
Cotyledonary Node	Control	0.00	0.00	0.00	0.00
	0.6	0.67	0.33	0.00	0.33
	0.8	0.33	0.00	0.00	0.11
	1.0	0.00	0.00	0.00	0.00
Mean		0.25	0.08	0.00	
Mature Embryo	Control	0.00	0.00	0.00	0.00
	0.6	0.67	0.67	0.00	0.45
	0.8	0.33	0.33	0.00	0.22
	1.0	0.00	0.00	0.00	0.00
Mean		0.25	0.25	0.00	
Grand Mean		0.25	0.14	0.00	
SE (m) ± (A)		0.17	0.14	-	
CD (5 percent) (A)		0.49	0.40	-	
SE (m) ± (B)		0.14	0.12	-	
CD (5 percent) (B)		0.42	0.35	-	
SE (m) ± (AxB)		0.29	0.24	-	
CD (5 percent) (AxB)		0.84	0.70	-	

(figures in the parenthesis are transformed values)

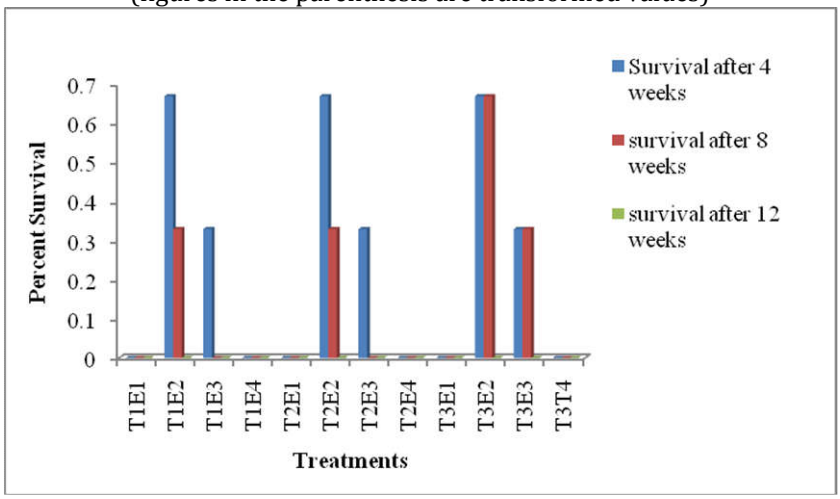

Fig. 2 Effect of bacterial cell density on transformation efficiency

Data from table 2 revealed that the effects of bacterial cell density with explants are significant for percent shoot survival after 4 weeks and 8 weeks on selection medium and 0.6 bacterial cell density was found best with all the three explants under study.

Figure 2 clearly showed that survival was constantly reduced after 8 weeks on selection medium in all the explants but highest in case of mature embryo when infected with culture having cell density 0.6 OD.

Mogali in 2005 co-cultivated cotton explants in different concentration of bacterial suspension (O.D. measured at 600 nm) at which the initial cell density of 0.5×10^8 cells/ml, wherein the explants survived without yellowing and death. Survival of explants was maximum (47) when bacterial culture of 0.2 O.D was used for co-cultivation. Although survival of explants was less (38) when bacterial culture of 0.6 O.D was used, a single kanamycin resistant shoot was obtained at this inoculum density (0.5×10^8 cells/ml) hence standardized as 0.6 [15]. The maximum number of putative transformants surviving green shoots was obtained by Kanthi in 2006 when *Agrobacterium* culture with 0.6 OD (18.33 percent) was used followed by 1.5 OD (11.03 percent.). The number of surviving plants was low at 2.0 OD (1.66 percent). Interaction effect between genotype and OD value were found significant. Asha at 0.6 OD value produced maximum putative transformants (20.3 percent) followed by cultivar Maruti at 1.5 OD (13.3 percent). The number of putative transformants produced was very low in cultivar Asha at 2.0 OD value (0.76 percent) [16].

Colonization Duration (Infection duration)

In order to obtain putative transformants it is necessary to infect explants with *Agrobacterium* culture for optimum period. The influence of duration of colonization for 15 min, 25 min and 30 min in dark was studied and explants without colonization was considered as control which showed no survival on selection medium.

Explants should be colonized for the optimum period of time in order to achieve appropriate gene integration along with minimum *Agrobacterium* overgrowth due to prolonged colonization. The colonization should be carried out in dark for the better integration of gene of interest into plant genome.

Table 3: Effect of colonization duration on transformation efficiency

Explants	Colonization Duration	Percent survival on selection medium			Mean
		4 weeks	8 weeks	12 weeks	
Shoot Tip	Control	0.00	0.00	0.00	0.00
	15 min.	0.67	0.33	0.00	0.33
	25 min.	0.33	0.00	0.00	0.11
	35 min.	0.33	0.00	0.00	0.11
Mean		0.33	0.08	0.00	
Cotyledonary Node	Control	0.00	0.00	0.00	0.00
	15 min.	0.67	0.33	0.00	0.33
	25 min.	0.33	0.00	0.00	0.11
	35 min.	0.33	0.00	0.00	0.11
Mean		0.33	0.08	0.00	

Mature Embryo	Control	0.00	0.00	0.00	0.00
	15 min.	1.00	0.67	0.00	0.56
	25 min.	0.67	0.33	0.00	0.33
	35 min.	0.33	0.00	0.00	0.11
Mean		0.50	0.25	0.00	
Grand Mean		0.83	0.14	0.00	
SE (m) ± (A)		0.19	0.13	-	
CD (5 percent) (A)		0.55	0.39	-	
SE (m) ± (B)		0.16	0.12	-	
CD (5 percent) (B)		0.48	0.34	-	
SE (m) ± (AxB)		0.33	0.23	-	
CD (5 percent) (AxB)		0.96	0.67	-	

(figures in the parenthesis are transformed values)

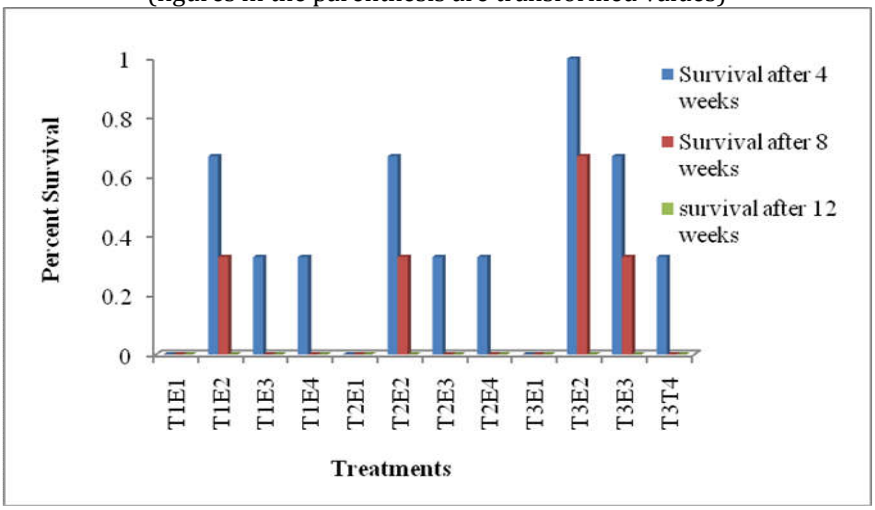

Fig. 3 Effect of colonization duration on transformation efficiency

Data from table 3 revealed that the effects of colonization duration with explants are significant for percent shoot survival after 4 weeks and 8 weeks on selection medium and colonization duration for 15 min was found best with all the three explants under study. Figure 3 showed that in case of shoot tip and cotyledonary node the survival was lower than mature embryo but when all the explants colonized for 15 min, the survival recorded was highest amongst the treatments.

The influence of duration of infection for 30 min and 60 min in shoot apical meristems of two cotton genotypes namely, Sahana and BC-68-2 belonging to *G. Hirsutum* was studied and it was observed that percent survival of explants was more when the infection period was 30 min (53.8 percent), when compared to 60 min (42.4 percent)[15]. Sangannavar in 2008 obtained significantly more number of plants survived in DLSa-17 (42) in 15 minutes of infection period than 30 minutes (38). Similarly, in SBYF-425, significantly higher numbers of plants (36) were obtained in 15 minutes of infection than 30 minutes (32)[20]. In Jayadhar, maximum number of plants were obtained when explants were infected with *Agrobacterium* for 10 min (72.5 percent) followed by 30 min (67.5 percent) of colonization. In Surabhi, regeneration of explants were maximum, when explants were infected with *Agrobacterium* for 10 min (73.75 percent) followed by 30 min (67.5 percent) of colonization [21].

Co-cultivation Duration

At the end of infection period, the explants were blot dried by using sterile blotters and placed back on the same shoot induction media for co-cultivation for different duration in dark so that the gene of interest from Agrobacterium should stably integrated into plant genome.

Co-cultivation duration should be optimum since less co-cultivation duration may prevent gene integration and more co-cultivation duration causes death of explants due to softening which may be due to increased moisture generated because of *Agrobacterium* overgrowth. Therefore, various co-cultivation durations were tried with all the three explants viz. 2 days, 3 days, 4 days and 5 days. The explants without co-cultivation served as control. When all the explants were co-cultured for 5 days, not single explants showed survival on selection medium at early stages of selection.

Table 4: Effect of co-cultivation duration on transformation efficiency

Explants	Co-cultivation duration	percent survival on selection medium			Mean
		4 weeks	8 weeks	12 weeks	
Shoot Tip	Control	0.33	0.00	0.00	0.11
	2 days	1.00	0.33	0.00	0.44
	3 days	0.67	0.33	0.00	0.33
	4 days	0.00	0.00	0.00	0.00
	5 days	0.00	0.00	0.00	0.00
Mean		0.40	0.13	0.00	
Cotyledonary Node	Control	0.67	0.00	0.00	0.22
	2 days	1.00	0.67	0.00	0.55
	3 days	0.67	0.33	0.00	0.33
	4 days	0.33	0.00	0.00	0.11
	5 days	0.00	0.00	0.00	0.00
Mean		0.53	0.20	0.00	
Mature Embryo	Control	0.67	0.33	0.00	0.33
	2 days	1.67	1.00	0.00	0.89
	3 days	1.33	0.67	0.00	0.66
	4 days	0.67	0.33	0.00	0.33
	5 days	0.00	0.00	0.00	0.00
Mean		0.87	0.47	0.00	
Grand Mean		0.60	0.27	0.00	
SE (m) ± (A)		0.18	0.18	-	
CD (5 percent) (A)		0.53	0.53	-	
SE (m) ± (B)		0.14	0.14	-	
CD (5 percent) (B)		0.41	0.41	-	
SE (m) ± (AxB)		0.32	0.32	-	
CD (5 percent) (AxB)		0.92	0.92	-	

(figures in the parenthesis are transformed values)

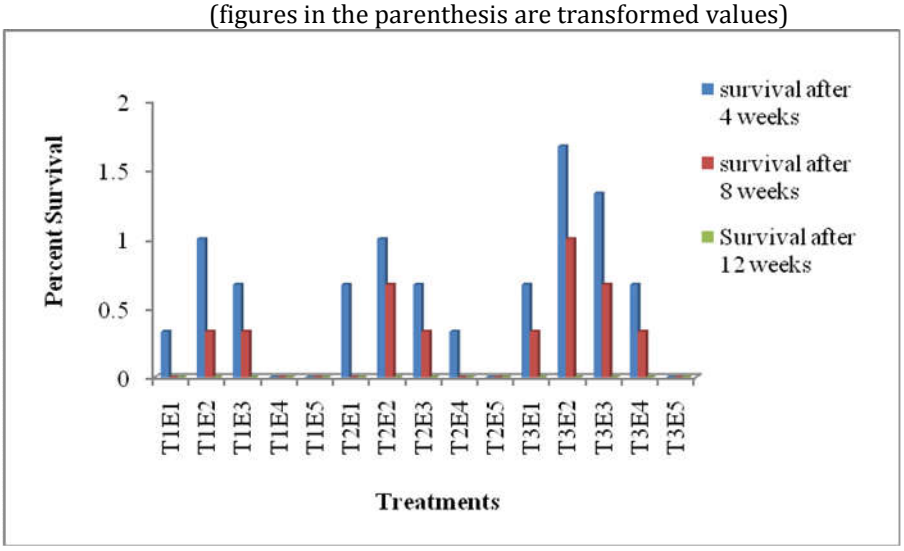

Fig 4 Effect of co-cultivation duration on transformation efficiency

Mature embryo showed somewhat higher mean percent survival than cotyledonary node and shoot tip which was 0.87 percent and 0.47 percent after 4 weeks and 8 weeks on selection media and no survival recorded after 12 weeks on selection medium.

A comparison based on mean performance of all the explants at every stage of selection irrespective of the co-cultivation duration showed that the percent survival was higher in case of mature embryo than the other two explants used in the study. Table 4 revealed that the effect of co-cultivation duration with explants are significant for percent shoot survival after 4 weeks, 8 weeks, 12 weeks on selection medium

and on rooting media, and co-cultivation for 2 days showed higher survival with all the three explants under study therefore used for further study.

From the figure 4 it is clear that the highest survival was recorded in case of mature embryo when co-cultivated for 2 days.

Finally, after completion of experiment for standardization of different parameters of transformation following parameters are set as standardized. For colonization, the *Agrobacterium* culture of 0.6 cell density was used with 100 µM of acetosyringone. The colonization duration was optimized to 15 min in dark and co-cultivated for 2 days in dark.

Although the effect of co-cultivation for 24 hrs and 48 hrs was observed statistically on par (80 percent and 76 percent respectively)[15], when the explants were co-cultivated with *Agrobacterium* for less than 48 hrs, percent survival on antibiotic media was less, hence the optimum time period for co-cultivation is determined to be 48 hrs. Highest plant survivability was observed in 48 hrs of co-cultivation that is 71.5 per cent in Jayadhar and 75 per cent in Surabhi. 48 hrs of co-cultivation recorded significantly higher regeneration than 72 hrs and 96 hrs of co-cultivation in both the genotypes [21]. Shoot apices were obtained after 48 hrs of preculture on 0.1 mg/lit BAP and 0.01 mg/lit NAA colonized for 15 minutes in *Agrobacterium tumefaciens*, EHA-105 were subjected to co-cultivation for 24 hrs, 48 hrs and 72 hrs in dark at 22^0C in basal MS medium supplemented with 0.1 mg/lit BAP and 0.01 mg/lit NAA followed by washing in cefotaxime, and culturing on MS medium supplemented with cefotaxime (400 mg/lit) and 0.1 mg/lit BAP and 0.01 mg/lit NAA, incubating at 28 ^{0}C ± 2 ^{0}C under light. The co-cultivation for 48 hrs showed higher survival of explants whereas at 72 hrs of co-cultivation shoot apices get soften and died [20].

CONCLUSION

Optimization of various parameters for increasing transformation efficiency may provide the opportunity for high frequency of transgenic development and genetic enhancement in cotton cultivar. These parameters may be used as a support for optimization of protocol for other genotypes of cotton in future.

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CITATION OF THIS ARTICLE

D A Padole, Gahukar S.J, Amrapali A. Akhare. Enhancement of Transformation efficiency using *Agrobacterium* in *Gossipium hirsutum* (L.) through parameters optimization. Bull. Env. Pharmacol. Life Sci., Vol 6[2] January 2017: 74-82