International Journal Of

Recent Scientific Research

ISSN: 0976-3031

Volume: 7(1) January -2016

AN ESTABLISHMENT OF EFFICIENT AGROBACTERIUM MEDIATED TRANSFORMATION IN TOMATO (*SOLANUM LYCOPERSICUM*)

Rajesh N., Siva Kumar J., John Elia Prashanth P and Osman Basha P

THE OFFICIAL PUBLICATION OF
INTERNATIONAL JOURNAL OF RECENT SCIENTIFIC RESEARCH (IJRSR)
http://www.recentscientific.com/ recentscientific@gmail.com

ISSN: 0976-3031

Available Online at http://www.recentscientific.com

International Journal of Recent Scientific Research
Vol. 7, Issue, 1, pp. 8583-8591, January, 2016

International Journal of Recent Scientific Research

RESEARCH ARTICLE

AN ESTABLISHMENT OF EFFICIENT *AGROBACTERIUM* MEDIATED TRANSFORMATION IN TOMATO (*SOLANUM LYCOPERSICUM*)

Rajesh N., Siva Kumar J., John Elia Prashanth P and Osman Basha P*

Department of Genetics and Genomics, School of Life Sciences, Yogi Vemana University Kadapa, AP, India

ARTICLE INFO

Article History:

Received 16th October, 2015
Received in revised form 24th November, 2015
Accepted 23rd December, 2015
Published online 28th January, 2016

Key words:

*Agrobacterium*mediated transformation, Vacuum Infiltration, cv. Arka Vikas.

ABSTRACT

An efficient *Agrobacterium* mediated genetic transformation protocol for the tomato (*Solanum lycopersicum*) cv. Arka Vikas was established. A comparison was made between hypocotyls and cotyledon on different regeneration media with and without *Agrobacterium* infection. Explants formed high-quality callus and regenerated on different types of media without *Agrobacterium* infection and kanamycin selectable media. But explants co-cultivated with *Agrobacterium tumefaciens* and in presence of kanamycin selection media, the callus induction and regeneration capability got reduced significantly. From these experiments by using cotyledonary explants an efficient protocol was developed for tomato using *Agrobacterium tumefaciens* mediated transformation. The transformation frequency was assessed in response to several different factors that include age of the explants, different types of explants (hypocotyls & cotyledons), three different *Agrobacterium* strains, infection time, co-cultivation time, initial kanamycin concentration during selection, different media combinations and different types of transformation methods. Eight day old cotyledonary explants were pre-cultured for 48 hrs on BAP (2 mg/l) and co-cultivated with *Agrobacterium* C58C1 harbouring P^{BIN} plasmid was vacuum infiltrated for 5 min at 25 Hg pressure followed by incubation on BAP (2 mg/l) and Indole acetic acid (0.1 mg/l) for 48 hrs in dark, and transferred on zeatin (2 mg/l) and Indole acetic acid (0.1 mg/l) regeneration media with kanamycin as to create selection pressure. Maximum root induction from regenerated shoots was achieved at half strength MS media. The developed protocol showed 60.61 ± 0.5% efficiency of transformation for tomato cultivar Arka Vikas. The standardized transformation protocol is simple, efficient and does not require tobacco, petunia, tomato feed layers.

INTRODUCTION

Agrobacterium mediated transformation**,** micro projectilebombardment, microinjection and electroporation are the most important transformation methods used for the development of transgenic plants. Out of these *Agrobacterium* mediated genetic transformation technique is comparatively less expensive, very effective and remains the most successful gene transfer methods in plants. Because of its advantages of low copy number, defined and preferential integration of transgene into transcriptional active regions in the chromosome and produced fertile transformed plants (Hiei *et al*, 2000; Kumar *et al*, 2011; Ling *et al*, 1998) and various insertional mutants were screened for mutants and characterized well for various phenotypes (Bhalla *et al*, 2009; Puri *et al*, 2010: Kumar *et al*, 2010). Abiotic and biotic stress resistance crops have also been developed through *Agrobacterium* mediated gene transformation technology (Wangxia *et al* 2003; Islam, 2006)**.**

A number of factors involved in genetic transformation greatly influence the overalltransformation efficiency (Hu and Phillips, 2001) and effect of various factors discussed in detaili.e. plant genotype (Frary and Earl, 1996, Park *et al*, 2003), explants type (Chyi and Phillips, 1987; Davis *et al*, 1991; Hamza and Chapeau, 1993; Park *et al*, 2003; Wu *et al*, 2006; Sun *et al*, 2006), plant growth regulators (Frary and Earle, 1996; Park *et al*, 2003; Cortina and Culianez-Macia, 2004; Wu *et al*, 2006; Sharma *et al*, 2009), pH in co-cultivation medium (Vernade *et al*, 1988; Gao *et al*, 2009 ; Wu *et al*, 2006), use of feeder layer (Qiu *et al*, 2007)and the *Agrobacterium*strain as well as its density in the inoculum (Davis *et al*, 1991; Wu *et al*, 2006; Islam 2007; Gao *et al*, 2009), phenolic compounds (Sun *et al*, 2006), infection and co-cultivation duration (Fillatti *et al*, 1987; Hanmza and chapeau, 1993, Park *et al*, 2003; Cortina and Culianeaz Macia, 2004).

Corresponding author:* **Osman Basha P
Department of Genetics and Genomics, School of Life Sciences, Yogi Vemana University Kadapa, AP, India

Tomato belongs to Solanaceae family and it is economically important vegetable crop consumed all over the world (Muller *et al,* 2005). Among dicotyledonous crop plants, tomato is also a genetic model plant with relatively small DNA content (950Mb) (Arumuganathan and Earle, 1991). Fruit ripening process was well studied and established in tomato, whereas it is impossible to study it in *Arabidopsis* and rice. Tomato fruits are rich in vitamins (A and C), carotenoids, and lycopene (Bhatia *et al,* 2004), which has a beneficial effect against cancer, heart and neural disorders (Fuhrman *et al,* 1997; Giovannucci,1999; Suganuma *et al,* 2004). The wealth of information regarding genome sequencing and EST's sequencing including sequencing full length c-DNA and mutants induced by a variety of mutagenic treatments, such as the application of EMS (Rajesh *et al,* 2014; Emmanuel and Levy, 2002; Menda *et al,* 2004) and all these germplasm can be used by using different screening procedure for salt stress (Basha *et al,* 2015), drought stress (Basha *et al,* 2015), yield, quality and quantity related mutants.

The first report of tomato transformation was published by Mc cormick *et al.*,1986, since then there has been numerous publications on transformation efficiency values of various tomato cultivars i.e, 8% (Vidya *et al,* 2000), 7-37% (Ling *et al,* 1998*)*, 9% (Roekel *et al,* 1993), 11% (Frary and Earle, 1996), 14% (Hamza and chapeau 1993), 20% (Park *et al,* 2003) and 28-48% (Sun *et al,* 2006) and 41.4% (Sharma *et al,* 2009). Standardization of tomato transformation procedure is still incomplete as different tomato cultivars vary in their response to various parameters. Hence development of an effective genotype independent tomato transformation method is crucial. In the present study tomato transformation protocol was standardized by taking care of different parameters i.e. age of the explants, types of explants (hypocotyls and cotyledons), *Agrobacterium* strains, infection time, co-cultivation time, kanamycin concentration, cefotaxime concentration and various hormonal combinations. *Agrobacterium* vacuum infiltration technique was established to develop highly efficient transformation protocol for the development of tomato transgenics.

MATERIALS AND METHODS

Plant material

Solanum lycopersicum, cultivar Arka Vikas seeds were obtained from Indian Institute of Horticulture Research (IIHR), Bangalore.

Bacterial strains

Three different strains harbouring plasmids were used for transformation of tomato. *AG1*strain with *PMDC100* vector and *C58C1* strain with P^{BIN}vector were grown in YEP media with Rifampicin (34 mg/l) and Kanamycin (50 mg/l) respectively. *LBA4404* strain harbouring PBI^{121}vectorgrown in YEM media with Streptomycin (100 mg/l) and Kanamycin (50 mg/l) supplement. Cells were pelleted at 28°C and 5000 rpm followed by washing with half-strength MS broth washing medium for two times and suspend the pellet in 10ml of washing medium. Bacterial cell density with 0.5 X 10^8 was used as final working concentration by diluting it with washing medium.

Sensitivity of tomato explants to kanamycin

The effect of the kanamycin on regeneration of explants was screened for the selection of transgenic plants. Cotyledons and hypocotyls explants were placed in different concentrations of kanamycin (20, 25, 30, 35, 40, 45, 50, 75 and 100 mg/l) with minimum number of 100 explants on each concentration. Explants were incubated in dark conditions for 2 weeks at 28°C and then incubated at 16/8 hrs (light/dark) photoperiod of 80 mol.m/2s/1 at 28°C conditions. The number of regenerated shoots was recorded after 5 weeks.

Cefotaxime effect on callus growth and shoot regeneration

To examine the influence of cefotaxime on callus growth and shoot regeneration, 100 cotyledonary explants were cultured on MS medium containing 0, 50, 100, 200, 250, 300, 350, 400, 450, 500, 550 and 600 mg/l cefotaxime. Regeneration frequency was calculated as a percentage of the number of regenerated explants/total number of explants (Ling *et al,* 1988).

Culture media

The composition of various media is described in components of Murashige and Skoog (MS) medium (Murashige and Skoog 1962), antibiotics and plant growth hormones were purchased from Himedia and stored at the prescribed temperature. Media components were mixed and pH was adjusted to 5.8 using 1M NaOH prior to addition of 0.7% w/v plant tissue-culture grade agar powder (Himedia Laboratories Pvt. Ltd. Mumbai, India) and then autoclaved. The growth hormones BAP and zeatin were dissolved in Dimethyl sulphoxide (DMSO) and IAA was dissolved in absolute ethanol. The antibiotics kanamycin and cefotaxime were prepared in MQ (Milli pore Corporation, USA) water and filter sterilized, whereas rifampicin was dissolved in methanol.

Table 1 Media composition used in various transformation and regeneration experiments

Component	Seed germination	Pre-culture	Co-cultivation	Washing	Selection	Rooting
MS salts	0.5x	1x	1x	0.5x	1x	1x
B5 Vitamins	0.5x	1x	1x	0.5x	1x	1x
Sucrose (g/lit)	15.0	30.0	30.0	15.0	30.0	30.0
Agar (%w/v)	0.7	0.7	0.7	-	0.7	0.7
BAP(mg/lit)	-	2	2	-	-	-
Zeatin (mg/lit)	-	-	-	-	2	-
IAA (mg/lit)	-	-	0.1	-	0.1	-
Kanamycin (mg/lit)	-	-	-	-	100	100
Coefataxime (mg/lit)	-	-	-	-	350	350

Antibiotics and growth hormones were added to the autoclaved medium after it was cooled to 50°C. Different media used for germination, pre-culture, co-cultivation, regeneration and rooting were shown in Table 1.

Agrobacterium mediated Transformation

Seeds of *Solanum lycopersicum* cv. Arka Vikas were surface sterilized in 4% (v/v) sodium hypochlorite solution with one drop of Tween-20 for 10 min and then rinsed for three times in sterilized distilled water. The seeds were germinated in a tissue culture bottle in germination media. All cultures were maintained at 28°C ± 2°C under 16h light/8h dark photoperiod with a light intensity of 60 µmol·m/2s/1. For *Agrobacterium* mediated genetic transformation, cotyledonary leaves with 1 to 2 mm in length and hypocotyls were used. To examine the influence of age of the explants on transformation efficiency, 100 cotyledonary explants (25 pieces per petridish) of age each 5^{th}, 6^{th}, 7^{th}, 8^{th}, 9^{th}, 10^{th} were infected and regenerated. Cotyledons were cut at the tip as well as at the base and middle segments of approximately 0.5 to 0.8 x 1.0 cm in size were placed on preculture medium. Transformation frequency was also evaluated at different infection periods of 5, 10, 15, 20, 25, 30 minutes and then co-cultivated for 24, 48, 72, 96 hrs.

Explants that showed regeneration or callus formation were sub-cultured into fresh selection medium for every 15 days. Regenerated shoots were excised from the callus and transferred into the rooting medium. Plantlets attained good shoot development and produced roots were transferred to pots containing coconut peat. Pots were kept in a humidity chamber for 3-5 days in the culture room under a 16/8 hrs light and dark cycle at 28°C and then in the green house at 28°C ± 2°C. The transformation efficiency was calculated as the percent co-cultivated explants producing independent transformation events, leading to regeneration of a complete plant on the kanamycin selection medium. Multiple shoots regenerated from a single callus were treated as a single transformation event.

PCR conditions used for kanamycin gene amplification

PCR was carried out in Thermal Cycler (Bio-Rad T100) with 1X PCR buffer (10 mM Tris, 5 mM KCl, 1.5 mM $MgCl_2$, 0.1% (w/v) gelatin, 0.005% (v/v) Tween-20, 0.005% (v/v) Np-40, pH 8.8), 0.2 m Md NTPs, 0.2 µl *Taq* polymerase and 5p moles of primers (FP-TGAATCCAGAAAAGCGGCCA; RP-ATTCGGCTATGACTGGGCAC) and 20 ng of genomic DNA. The PCR cycling conditions included initial denaturation for 4 min at 94°C, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 45 sec, and extension at 72°C for 1 min, and followed by a final extension at 72°C for 8-10 min. The amplified PCR products were resolved on 1.5% (w/v) agarose gel (Himedia) in 1X TAE buffer in electrophoresis unit (Japan) for 1-2- hours at 75V.

RESULTS

Age of the explants

It is well established that the age of the explants greatly influences the transformation and regeneration potential. All ages of cotyledons (5-10 days) achieved transformation and significant differences were noted among different ages of explants (Fig 1). Maximum transformation frequency was observed at 8 days old seedlings (Fig 1).

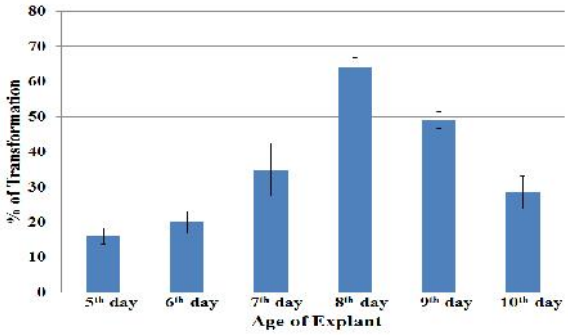

Figure 1 Effect of age of the explants on transformation frequency

Type of the Explants

The cotyledon explants showed high callus formation and regeneration during transformation. The cotyledon explants recovered kanamycin resistant callus regeneration during subsequent weeks was high in comparison to hypocotyls (Fig2).

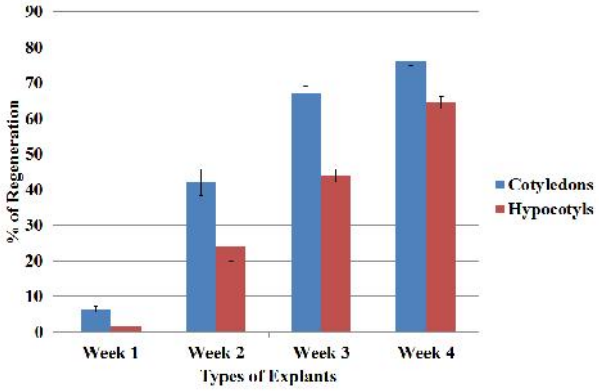

Figure 2 Effect of nature of explants on transformation frequency

Effect of bacterial density, infection time and co-cultivation period on transformation frequency

Bacterial strains *AG1*, *LBA4404*, *C58C1* with bacterial densities of 0.5, 1.0 and 2.0 were used for infection with infection period of 5, 10, 15 and 20 min with different co-cultivation time of 24, 48, 72, 96 hrs. Efficient T-DNA transformation was achieved at the following conditions; the *AG1* strain with 0.5 X 10^8 cells/ml density with infection period of 5 min and with co-cultivation period of 24 hrs, the *LBA4404* strain with 0.5 X 10^8 cells/ml with infection time of 10 min and co-cultivation period of 48 hrs, and for *C58C1* strain with 1.0 X 10^8 cells/ml with infection time of 15 min and co-cultivation period of 48 hrs by maintaining vacuum pressure of 25 Hg.

Callus started developing from the co-cultivated explants in the second week and shoots were developed after 4-6 weeks co-culture on selection medium containing antibiotics and growth regulators. Pre-cultured control explants which did not receive co-cultivation died on the selection medium after three weeks of their transfer to the selection medium. *AG1* strain with

bacterial density i.e. 0.5, 1.0, 2.0 OD and infection time of about 10, 15 and 20 min were checked and found that at higher density (1.0 and 2.0 OD) with co-cultivation period for 48, 72, 94 hrs produced lower transformation frequency by an overgrowth of *Agrobacterium* and necrotic lesions on the explants by both methods of transformation. (Fig.3 and Tables 2&3) Similarly for *LBA4404* strain 1.0 and 2.0 OD and infection time of 15&20 min with co-cultivation period for 72, 94, hrs produced lower transformation efficiency by both methods of transformation (Fig 3, Tables 2 and 3). *C58C1* with optical density of 2.0 was used with infection period of about 20 min with co-cultivation time for 72, 94 hrs produced lower transformation efficiency by both methods of transformation (Fig 3, Tables 2 and 3).

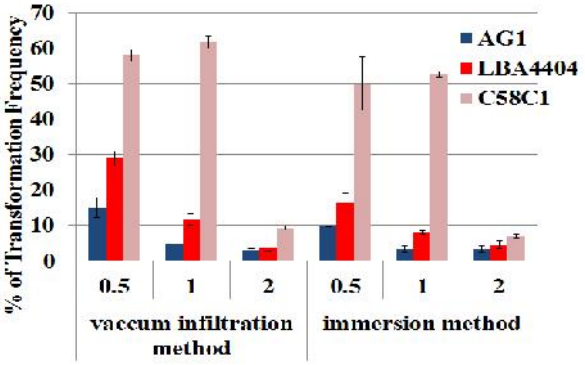

Figure 3 Effect of *Agrobacterium* Bacterial density on Transformation frequency

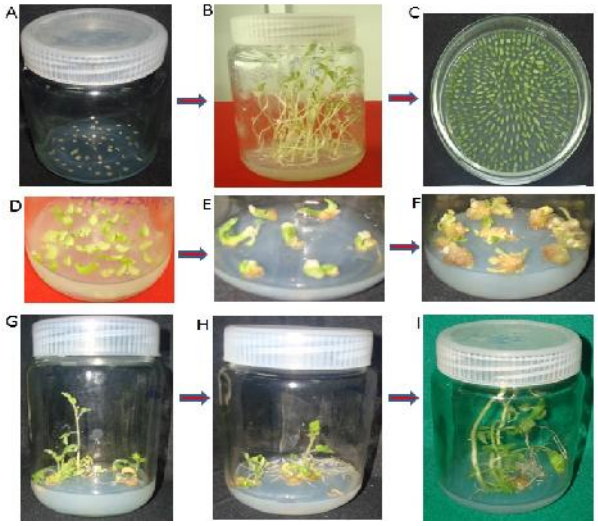

Figure 4 Different stages of *Solanum lycopersicum* cv. Arka vikas transformation.

(A) Seed germination on germination medium. (B) Fully expanded cotyledonary leaves after 8-days of germination. (C) Cotyledonary explants on pre-culture medium. (D) Cotyledonary explants on selection medium after co-cultivation under vacuum infiltration for 5 min at 25 Hg pressure with *Agrobacterium* harbouring the PBIN binary vector. (E) Callus with regenerating shoot buds growing on selection medium. (F) Multiple shoots regenerating from a single callus. (G) Growing shoots on callus. (H) Young plantlet developing roots on selection medium. (I) Mature transgenic tomato plant.

Infecting the explants with high bacterial density and prolonged infection time and co-cultivation the explants could not be rescued as all the explants died due to over growth of *Agrobacterium*. The average transformation frequency for *AG1*, *LBA4404*, and *C58C1* by vacuum infiltration method was about 22.66%, 35%, and 60.61% respectively. Immersion method of *Agrobacterium* transformation efficiency was reduced to about 12%, 23.66%, and 52.66% respectively for *AG1*, *LBA4404* and *C58C1*. Among the used *Agrobacterium* strains *C58C1* strain produces more transformation frequency than other strains i.e. *AG1* and *LBA4404* in both the methods. Hence the transformation efficiency in ascending order of tomato cultivar Arka Vikas was *C58C1> LBA4404>AG1*.

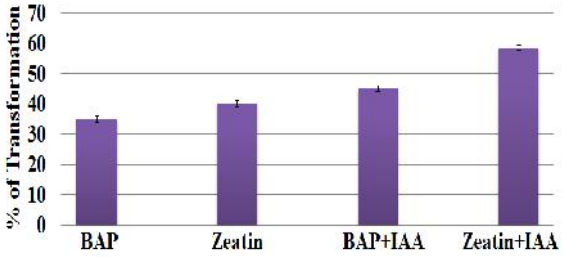

Figure 5 Effect of different types of regeneration media on transformation frequency

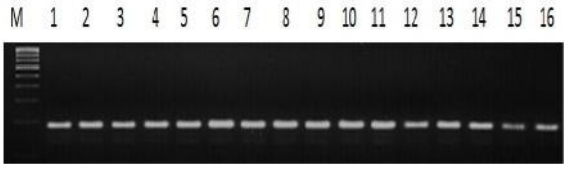

Figure 6 PCR amplification of kanamycin gene in transgenic Plants; M-1 Kb Ladder, Lane 1 to 16- insertional mutants.

Effect of different types of regeneration media on transformation efficiency

Compared with other types of media, zeatin + IAA combination showed more regeneration of explants. The effect of zeatin or BAP alone and in combination with IAA on transformation of Arka Vikas was investigated. It was observed that zeatin was more effective than BAP, as the use of the (2 mg/l) resulted in a transformation efficiency of 40% in this cultivar, whereas the use of BAP (2 mg/l) showed only 35% efficiency. Combination of the both BAP (2 mg/l) and IAA (0.1 mg/l) was found to increase the transformation efficiency to 42% than the BAP (2 mg/l) was used. But when the media supplemented with zeatin (2 mg/l) and IAA (0.1 mg/l) was found to improve the transformation efficiency to 58%.

Transgenic Plant Selection and Regeneration

The well-developed callus produced shoots are called as plantlets and were transferred on rooting medium. Kanamycin resistant shoots were transferred to fresh rooting medium after 15 days to sustain root growth. The well rooted transformed shoots were separated from the media and successfully hardened and acclimatized in the green house conditions and the plantlets were transferred to soil. Mature transgenic plants were found morphologically similar to wild type plants and they produced fruits.

Effect of kanamycin

In the present study we found that, kanamycin at the concentration of 30 mg/l completely inhibited the callus formation in the non-transformed explants.

Table 2 Effect of infection time on transformation frequency, Data are mean ± SE of 3 replicates.

Transformation method	Infection time (min)	Number of explants infected	Mean number of transgenic plants produced AG1 at 0.5 OD	Mean number of transgenic plants produced LBA4404 at 0.5 OD	Mean number of transgenic plants produced C58C1 at 1.0 OD
Vacuum infiltration	5	100	23±1.5	26±3.7	28.66±3.17
	10	100	12.33±1.4	38.33±4.4	36.66±1.7
	15	100	7±1.52	12.66±3.7	55±2.88
	20	100	2±0.57	4.83±0.1	19±3.78
Immersion method	5	100	13±1.5	14±2.08	17±1.5
	10	100	8±0.57	24.66±4.66	23.33±3.33
	15	100	4.66±0.33	8.33±3.33	33.33±3.33
	20	100	2.33±0.33	4.33±1.85	8.33±2.72

Table 3 Effect of Co-cultivation Periods on transformation frequency, Data are mean ± SE of 3 replicates.

Transformation method	Co-cultivation Periods (hrs)	Number of explants infected	Mean number of transgenic plants produced AG1 at 0.5 OD	Mean number of transgenic plants produced LBA4404 at 0.5 OD	Mean number of transgenic plants produced C58C1 at 1.0 OD
Vaccum infiltration	24	100	22.66±1.4	14.33±0.33	33.66±2.40
	48	100	10.66±0.66	35±2.88	58.33±1.2
	72	100	4.41±0.36	14±3.05	24±0.57
	96	100	2.02±0.03	5.66±1.45	18±3
Immersion method	24	100	12.33±1.45	9.6±3.1	21.33±0.88
	48	100	7±1	23.66±0.88	37.66±3.92
	72	100	5±1.7	8.66±0.66	16.66±1.66
	96	100	1.33±0.33	2.33±0.88	8.33±2.02

Thus 30 mg/l kanamycin was found to be the minimum inhibitory concentration (MIC) for the selection of transformed callus initially.

Afterwards subsequent sub-culturing wat done in increased kanamycin concentration results in the selection of transformed adventitious shoot buds and rooting. Further, the transformed shoots were able to survive at 100mg/l kanamycin selection pressure, but all non-transformed shoots lost their green colour at the same concentration via etiolation.

PCR analysis of the transgenic plants

The insertional mutant seeds were checked for the presence of kanamycin gene and expected size of amplification was observed in the insertional mutants and no amplification was obtained in wild type (Fig 6).

DISCUSSION

The transformation efficiency of tomato depends upon many factors such as the cultivar, type of explants, age of the explants, *Agrobacterium* strain and its density, co-cultivation time and regeneration medium (Davis *et al*, 1991; Madhulatha *et al*, 2007).

Age and type of the explants

The higher frequencies of transformation efficiency observed for up to 8-days-old seedlings and it may be attributed to the fact that active plant cell metabolism is required for the synthesis of *vir* inducing factors leading to the effective plant transformation. Developing leaves have less differentiated and more metabolically active cells, and show improved plant regeneration responses, when provided with suitable hormonal and culture media conditions.

The majority of reports for tomato transformation suggest using cotyledons from 6 to 10 day old seedlings for transformation (Davis *et al.*, 1991; Frary and Earle, 1996; Park *et al*, 2003; Sun *et al*, 2006; Wu *et al*, 2007; Gao *et al*, 2009). Mazumdar *et al.* (2010) demonstrated that in the case of *Jatropha curcas*, the cotyledonary explants excised from freshly in-vitro germinated seedlings were the most amenable to *Agrobacterium* mediated transformation as compared to older explants.

The cotyledons explants showed better performance in regeneration of plantlets through callus than hypocotyls explants, demonstrated that the cotyledons are excellent explants for tomato plant regeneration. As it is well established that cotyledon explants show good regeneration in tomato (Fillati *et al*, 1987; Hamza and Chapeau, 1993; Frary and Earle, 1996; Ellul *et al*, 2003) these were used as explants for transformation. Previous studies demonstrated that cotyledons of tomato were superior to other sources of explants, including hypocotyls, stems and leaves for promoting shoot organogenesis of tomato (Hamza and Chapeau, 1993; Van Roekel *et al*, 1993; Ling *et al*, 1998).

Previous reports on tomato transformation suggerted the use of feed layer during pre-culture and *Agrobacterium* co-cultivation (McCormick *et al*, 1986; Fillatti *et al.*,1987; Roeken *et al*, 1993; Frary and Earle, 1996; Ling *et al*, 1998; Zhang and Blumwald, 2001; Cortina and Culianez-Macia, 2004; Frary and Van Eck, 2005), which makes the transformation protocol difficult. In our study, pre-culture (BAP 2mg/l), co-cultivation (2mg/l BAP) and (0.1 mg/l IAA) were done on basal medium of MS containing growth hormones, without feeder layers the same has been reported by Qiu *et al*, (2007) for cultivar Micro-Tom.

Effect of Kanamycin

Kanamycin is an effective aminoglycosidic antibiotic, which has been used as a successful selective agent of transformed

tomato plantlets ranges from 50 (Kaur and Bansal, 2010; Li *et al*, 2003; Riggs *et al*, 2001) to 100 mg/l (Li *et al*, 2003; Rai *et al*, 2013; Ying *et al*, 2008). In the present study we employed 30 mg/l kanamycin for initial selection, and increasing level of concentration of kanamycin from 50 mg/l to 100 mg/l for further selection of transformed shoots and it helps to prevent potentially shots escapes, and that verified the efficiency of kanamycin as a selection marker, as reported previously.

Effect of strain, growth regulators and vacuum infiltration

The effects of *Agrobacterium* bacterial density and growth regulators on the transformation efficiency and regeneration were studied. A bacterial density of 0.5 OD and co-cultivation time of 48 h resulted in the maximum number of independent transformation events. Most tomato transformation protocols demonstrated that explants on co-cultivation with various *Agrobacterium* strains (LBA 4404, C58C2, GV311SE or A208) for 48 h with bacterial densities ranging from 1.0 to 2.0, giving variable transformation efficiencies (8- 48%) with different cultivars of tomato (McCormick *et al*, 1986; Hamza and Chapeau, 1993; Lipp-Joao and Brown, 1993: Frary and Earle, 1996; Oktem *et al*.,1999; Vidya *et al*, 2000; Pozueta *et al*, 2001; Jia *et al*, 2002; Ellul *et al*, 2003). In our study, we observed that co-cultivation up to 48 h using different strains at a OD of 0.5 increased the transformation efficiency to 60.6% for variety Arka Vikas compared with 41.4% (Sharma *et al*, 2009).

There is a threshold range of a combination of bacterial concentration and time of infection. Beyond, increase in threshold level of bacterial optical density or infection time or co-cultivation time from 72 h effects the explants negatively by turning explants into black colour and finally losing their regeneration capacity. In addition to this, removal of excess bacterial solution from the explants before transfer to the co-cultivation medium is also important, because if a little bacterial suspension was present on explants; bacterial growth affects the health of the explants within 48 h. Transformation frequency obtained with zeatin alone at a bacterial concentration of 2 mg/l was found to be as good as that obtained with zeatin+IAA and was superior to that achieved by supplementing the regeneration medium with BAP alone or BAP in combination with IAA. This improved the transformation efficiency using zeatin in the regeneration medium, rather than BAP and the transformation efficiency was statistically significant. It has been reported earlier that zeatin in combination with IAA has improved the frequency of callus formation and shoot regeneration in tomato (Park *et al*, 2003). In our study, although the combined use of a cytokinin and IAA in the regeneration medium showed only a slight increase in the number of regenerated transgenic plants.

Small calli and shoot primordial were formed across the cotyledonary explants that were cut at base and tip and those infected with *Agrobacterium tumefaciens*. It has been reported that the wounding releases phenolics and aids in the release of ethylene gas which is believed to be involved in the formation of adventitious shoots from the wounded sites (George, 1993). Generally, wounding of explants during transformation provides a way for the *Agrobacterium* to travel deep into the tissue to infect the meristematic cells. Previously Charity *et al*, 2002, followed the vortex method for creating wounds across the cotyledon explants of *Pinus radiate*, and the half seed explants of soya bean were wounded by piercing with gauge needle (Chee *et al*, 1989) prior to *Agrobacterium* infection. As a result higher transformation efficiency was achieved. Developing an efficient transformation system is a pre-requisite to achieve higher transformation efficiency, the punctured cotyledon method was previously found effective for genetic manipulation of tomato to develop phenotypically normal and fertile transgenic plants (Shahin *et al*, 1986).

Efforts were made to increase the transformation frequency for the generation of transgenics by vaccum infiltration of explants with *Agrobacterium* suspension. This technique improves transformation frequency in a wide variety of plant species citrus and cowpea (de Oliveira *et al*, 2009; Bakshi *et al*, 2011). To improve access of *Agrobacterium* as well to create an area of wounding to induce cotyledonary cells and to produce phenolic compounds for *vir* gene induction vaccum infiltration method is the best method. We attempted various time intervals of vacuum infiltration of explants at 25 mmHg in an *Agrobacterium* suspension, and of the different time intervals tested, it has results a maximum of 60.6% transient transformation frequency. In vaccum infiltration technique regenerating cells of cotyledonary explants are positioned a few cells layers beneath the surface at the axils in *Vigna* species including *cowpea, mungbean and blackgram*. Vacuum infiltration of cotyledon explants of *Pinus radiata* in an *Agrobacterium* suspension has allowed *Agrobacterium* to penetrate several layers deep through the sub-epidermal layer to mesophyll cells and vascular tissues (Charity *et al*, 2002), although the cells buried several layers deep, were not necessarily those that would induce shoots (Yeung *et al*, 1981). The vacuum infiltration of *Agrobacterium* has been successfully used to produce transgenic plants of model plant *Arabidopsis* (Clough and Bent, 1998), and recalcitrant crop species like wheat (Cheng *et al*, 1997), mungbean (Jaiwal *et al*, 2001), pinus (Charity *et al*, 2002), cotton (Leelavathi *et al*, 2004), kidney bean (Liu *et al*, 2005), coffee (Canche- Moo *et al*, 2006), chickpea (Indurker *et al*, 2010) and banana (Subramanyam *et al*, 2011). The total time for vacuum infiltration was fixed at 10 min, as the same time period reported previously for immersion in *Agrobacterium* suspension (Rajagopalan and Perl-Treves, 2005; Selvaraj *et al*, 2010; Tabei *et al*, 1998). Increasing the vacuum infiltration treatment time beyond 15 min, the nodal explant's meristems were injured.

CONCLUSION

Developing an efficient transformation system is a pre-requisite to achieve higher transformation frequency for insertional mutagenesis programmes. Vaccum infiltration method of transformation was previously found effective for genetic manipulation to develop phenotypically normal and fertile transgenic plants. Thus to enhance the transformation frequency of tomato, we used the vacuum infiltration method. This method is simple and suitable system for high frequency transformation of tomato cultivars. The developed healthy transformed shoots have displayed the normal phenotype

suggesting that the cotyledonary explants as a suitable source for plant regeneration and transformation. We have also optimized the factors influencing transformation frequency such as *Agrobacterium* density, infection time and co-cultivation period. The transformation efficiency in the vacuum infiltration method was 60.6% which was higher than earlier transformation reports. This method of transformation approach might help to develop cultivars with high quality and quantity, insect resistance and stress resistance in tomato.

Acknowledgements

We express sincere thanks to Council of Scientific & Industrial Research (***CSIR***), R&D organisation (Sanction letter No. 38(1329)/12/EMR-II, dated 03-04-2012), India for the financial support.

References

Arumuganathan, K., and Earle, E. 1991. Estimation of nuclear DNA content of plants by flow cytometry; Plant Mol. Biol. Rep. 9 208–218.

Bakshi, S., Sadhukhan, A., Mishra, S., and Sahoo, L. 2011. Improved *Agrobacterium*-mediated transformation of cowpea via sonication and vacuum infiltration. Plant Cell Rep. 30:2281–2292.

Basha, P.O., Sudarsanam, G., Reddy, M.M.S., and Sankar, N.S. 2015. Effect of PEG Induced Water Stress on Germination and Seedling Development of Tomato Germplasm. IJRSR. 6 (5): 4044-4049.

Basha, P.O., Reddy, M.M.S., Riazunnisa, K., and Reddy, M.S. 2015. In Vitro Evaluation of Tomato Genotypes for Salt Tolerance at Seedling Stage. IJPAES. 5 (1): 102-106.

Bhalla, A., Basha, P.O.**,**Kumar, M., Singh, K., Rajpurohit, D., Randhawa, G.S., and Dhaliwal H.S. 2009. Linkage Mapping of Polyembryonic, Oligoculm and Gibberellic acid insensitive Dwarf Insertional Mutants of *Oryza sativa* Var. Basmati 370. SABRAO Journal of Breeding and Genetics. 41 (1): 13-23.

Bhatia, P., Ashwath, N., Senaratna, T., and Midmore, D. 2004. Tissue culture studies of tomato (*Lycopersicon esculentum*). Plant Cell Tissue Organ Cult. 78: 1–21.

Canche-Moo, R.L.R., Ku-Gonzalez, A., Burgeff, C., Loyola-Vargas, V.M., Rodrıguez-Zapata, L.C., and Castan˜o, E. 2006. Genetic transformation of Coffea canephora by vacuum infiltration. Plant Cell Tiss Org Cult. doi:10.1007/s11240-005-9036-4.

Charity, J.A., Holland, L., Donaldson, S.S., Grace, L., and Walter, C. 2002. *Agrobacterium* mediated transformation of *Pinus radiata* organogenic tissue using vacuum infiltration. Plant Cell Tissue Organ Cult. 70: 51–60.

Chee, P.P., Fober, K.A., and Slightom, J.L. 1989. Transformation of soybean (*Glycine max*) by infecting germinating seeds with *Agrobacteriumtumefaciens*. Plant Physiol. 91: 1212–1218.

Cheng, M., Fry, J.E., Zhou, H., Hironaka, C.M., Duncan, D.R., Coner, T.W., and Wan, Y. 1997. Genetic transformation of wheat mediated by *Agrobacteriumtumefaciens*. Plant Physiol. 115: 971–980.

Clough, J., and Bent, A.F. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. 16: 735–743.

Chyi, Y.S. and Phillips, G.C. 1987. High efficiency *Agrobacterium*-mediated transformation of Lycopersicon based on conditions favorable for regeneration. Plant Cell Rep. 6: 105-108.

Cortina, C., and Culianez-Macia, F.A. 2004. Tomato transformation and transgenic plant production. Plant Cell Tiss. Org. Cult. 76: 269–275.

Davis, M.E., Lineberger, R.D., and Miller, A.R. 1991. Effects of tomato cultivar, leaf age, and bacterial strain on transformation by *Agrobacteriumtumefaciens*. Plant Cell Tiss. Org. Cult. 24: 115–121.

De Oliveira, M.L.P., Febres, V.J., Costa, M.G.C., Moore, G.A., and Otoni, W.C. 2009. High-efficiency *Agrobacterium*-mediated transformation of citrus via sonication and vacuum infiltration. Plant Cell Rep. 28: 387–395.

Ellul, P., Garcia-Sogo, B., Pineda, B., Rios, G., et al. 2003. The ploidy level of transgenic plants in *Agrobacterium*-mediated transformation of tomato cotyledons (*Lycopersicon esculentum* Mill.) is genotype and procedure dependent. Theor. Appl. Genet.106: 231-238.

Emmanuel, E., and Levy, A.A. 2002. Tomato mutants as tools for functional genomics. Curr. Opin. Plant. Biol. 5: 112–117.

Fillati, J.J., Kiser, J., Rose, R., and Comai, L. 1987. Efficient transfer of a glyphosate tolerance gene into tomato using a binary *Agrobacterium tumefaciens* vector. Bio Technology. 5: 726–731.

Frary, A., and Van Eck, J. (2005). Organogenesis from transformed tomato explants. Methods Mol. Biol.286: 141-150.

Frary, A., and Earle, E.D. 1996. An examination of factors affecting the efficiency of *Agrobacterium*-mediated transformation of tomato. Plant Cell Rep. 16: 235–240.

Fuhrman, B., Elis, A., and Aviram, M. 1997. Hypocholesterolemic effect of lycopene and beta-carotene is related to suppression of cholesterol synthesis and augmentation of LDL receptor activity in macrophages. Biochem. Biophys. Res. Commun. 233: 658–662.

Gao, N., Shen, W., Cao, Y., Su, Y., and Shi, W. 2009. Influence of bacterial density during preculture on *Agrobacterium*-mediated transformation of tomato. Plant Cell Tissue Organ Cult. 98: 321–330.

George, E.F. 1993. Factors affecting growth and morphogenesis. In: George, E.F. (Ed.), Plant propagation by tissue culture. Exegetics Limited, London, pp. 231–271.

Giovannucci, E. 1999. Tomatoes, tomato-based products, lycopene, and cancer: review of the epidemiologic literature. J. Natl. Cancer Inst. 91: 317–331.

Hamza, S., and Chupeau, Y. 1993. Re-evaluation of conditions for plant regeneration and *Agrobacterium*-mediated transformation from tomato (*Lycopersicon esculentum*). J. Exp. Bot. 44: 1837–1845.

Hiei, Y., Komari, T., Ishida, Y., and Saito, H. 2000. Development of *Agrobacterium* mediated transformation method for monocotyledonous plants. Breed. Res. 2: 205–213.

Hu, W., and Phillips, G.C. 2001. A combination of overgrowth-control antibiotics improves *Agrobacterium tumefaciens*-mediated transformation efficiency for cultivated tomato (*L. esculentum*). In Vitro Cell. Dev. Biol. 37: 12–18.

Indurker, S., Misra, H.S., and Eapen, S. 2010. *Agrobcterium*-mediated transformation in chickpea (*Cicer arietinum L.*) with an insecticidal protein gene: optimization of different factors. Physiol Mol Biol Plants. 16(3): 273–284.

Islam. A. 2006. Fungus resistant transgenic plants: strategies, progress and lessons learnt. Plant Tissue Cult. & Biotech. 16(2): 117-138.

Jaiwal, P.K., Kumari, R., Ignacimuthu, S., Potrykus, I., and Sautter, C. 2001. *Agrobacterium tumefaciens*-mediated transformation of mungbean (*Vigna radiata*) - a recalcitrant grain legume. Plant Sci. 161: 239–247.

Jia, G.X., Zhu, Z.Q., Chang, F.Q., and Li, Y. 2002 Transformation of tomato with the BADH gene from Atriplex improves salt tolerance. Plant Cell Rep. 21: 141–146.

Kaur, P., and Bansal, K.C. 2010. Efficient production of transgenic tomatoes via *Agrobacterium*-mediated transformation. Biol. Plantarum. 54: 344–348.

Kumar, M., Basha, P.O., Puri, A., Rajpurohit, D., Randhawa, G.S., Sharma, T.R., Dhaliwal, H.S. 2010. Cloning of a candidate gene *OsAPC6* of anaphase promoting complex of rice through T-DNA insertion. Funct Integr Genomics, 10 (3): 349-358.

Kumar, R., Singh, B., Yadav, A., Goswami, A., and Sharma, A., 2011. The use of *Agrobacterium*-mediated transformation methodology in tomato (*Lycopersicon esculentum*): current trends and developments. Ann. Hortic. 4: 1–9.

Leelavathi, S., Sunnichan, S.G., Kumria, R., Vijaykanth, G.P., Bhatnagar, R.K., and Reddy, V.S. 2004. A simple and rapid *Agrobacterium* mediated transformation protocol for cotton (*Gossypium hirsutum* L.): Embryogenic calli as a source to generate large numbers of transgenic plants. Plant Cell Rep. 22: 465–470.

Li, H.Y., Chang, C.S., Lu, L.S., Liu, C.A., Chan, M.T., and Charng, Y.Y. 2003. Overexpression of *Arabidopsis thaliana* heat shock factor gene (AtHsfA1b) enhances chilling tolerance in transgenic tomato. Bot. Bull. Acad. Sinica. 44: 129–140.

Ling, H.Q., Kriseleit, D., and Ganal, M. 1998. Effect of ticarcillin/potassium clavulanate on callus growth and shoot regeneration in *Agrobacterium*-mediated transformation of tomato (*Lycopersicon esculentum* Mill.). Plant Cell Rep. 17: 843–847.

Lipp Joao, K.H., and Brown, T.A. 1993. Enhanced transformation of tomato co-cultivated with *Agrobacterium* tumefaciens C58C1Rifr::pGSFR1161 in the presence of acetosyringone. Plant Cell Rep. 12: 422–425.

Liu, Z., Park, B.J., Kanno, A., and Kameya, T. 2005. The novel use of a combination of sonication and vacuum infiltration in *Agrobacterium*- mediated transformation of kidney bean (*Phaseolus vulgaris* L.) with lea gene. Mol Breed. 16: 189–197.

Madhulatha, P., Pandey, R., Hazarika, P., and Rajam, M.V. 2007. High transformation frequency in *Agrobacterium*-mediated genetic transformation of tomato by using polyamines and maltose in shoot regeneration medium. Physiol. Mol. Biol. Plants. 13: 191–198.

Mazumdar, P., Basu, A., Paul, A., Mahanta, C., and Sahoo, L. 2010. Age and orientation of the cotyledonary leaf explants determine the efficiency of de novo plant regeneration and *Agrobacterium tumefaciences*-mediated transformation in *Jatropha curcas* L. South African Journal of Botany. 76: 337-344.

McCormick, S., Niedermeyer, J., Fry, J., Barnason, A., Horsch, R., and Fraley, R. 1986. Leaf disc transformation of cultivated tomato (*L. esculentum*) using *Agrobacterium tumefaciens*. Plant Cell Rep. 5: 81–84.

Menda, N., Semel, Y., Peled, D., Eshed, Y., and Zamir, D. 2004. In silico screening of a saturated mutation library of tomato. Plant J. 38: 861–872.

Mueller, L.A., Tanskley, S.D., Giovannoni, J.J., van Eck, J., Stack, S., Choi, D., Kim, D., Chen, M., et al. 2005. The tomato sequencing project, the fi rst corner stone of the international Solanaceae project (SOL). Comp. Funct. Genomics. 6: 153–158.

Murashige, T., and Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 15: 473–497.

Oktem, H.A, Bulbul, Y., Oktem, E., and Yucel, M. 1999. Regeneration and *Agrobacterium*-mediated transformation studies in tomato (*Lycopersicon esculentum* Mill). Tr. J. Bot. 23:345–348.

Park, S.H., Morris, J.L., Park, J.E., Hirschi, K.D., and Smith, R.H. 2003. Efficient and genotype-independent *Agrobacterium*-mediated tomato transformation. J. Plant Physiol. 160: 1253–1257.

Pozueta-Romero, J., Houlne, G., Ca˜nas, L., Schantz, R., and Chamarro, J. 2001. Enhanced regeneration of tomato and pepper seedling explants for *Agrobacterium* mediated transformation. Plant Cell Tissue Organ Cult. 67: 173–180.

Puri, A.**,** Basha, P.O., Kumar, M., Rajpurohit, D., Randhawa, G.S., Kianian, S.F., Rishi, A., and Dhaliwal, H.S. 2010. The polyembryo gene (*OsPE*) in rice. Funct Integr Genomics: 10 (3): 359-366.

Qiu, D., Diretto, G., Tavarza, R., and Giuliana, R. 2007. Improved protocol for *Agrobacterium* mediated transformation of tomato and production of transgenic plants containing carotenoid biosynthetic gene Cs ZCD. Sci. Hortic. 112: 172-175.

Rai, A.C., Singh, M., and Shah, K. 2013. Engineering drought tolerant tomato plants over-expressing BcZAT12 gene encoding a C2H2 zinc finger transcription factor. Phytochem. 85: 44–50.

Rajagopalan, P.A., and Perl-Treves, R. 2005. Improved cucumber transformation by a modified explant dissection and selection protocol. HortScience. 40:431–435.

Rajesh, N., Prashanth, P.J.E., and Basha, P.O. 2014. Development of a Mutant Genetic Resource of Tomato (*Solanum lycopersicum* L.) cv. ArkaVikas. Annals of Biological Research. 5 (11): 1-6.

Riggs, C.D., Zeman, K., DeGuzman, R., Rzepczyk, A., and Taylor, A.A. 2001. Antisense inhibition of a tomato meiotic proteinase suggests functional redundancy of proteinases during microsporogenesis. Genome. 44: 644–650.

Roekel, J.S.C., Damm, B., Melchers, L.S., and Hoekema, A. 1993. Factors influencing transformation frequency of tomato (*Lycopersicon esculentum*). Plant Cell Rep. 12: 644–647.

Selvaraj, N., Kasthurirengan, S., Vasudevan, A., Manickavasagam, M., Choi, C.W., and Ganapathi, A. 2010. Evaluation of green fluorescent protein as a reporter gene and phosphinothricin as the selective agent for achieving a higher recovery of transformants in cucumber (*Cucumis sativus* L. cv. Poinsett76) via *Agrobacterium tumefaciens*. In Vitro Cell Dev Biol Plant. 46: 329–337.

Shahin, E.A., Sukhapinda, K., Simpson, R.B., and Spivey, R. 1986. Transformation of cultivated tomato by a binary vector in *Agrobacterium rhizogenes*: transgenic plants with normal phenotypes harbor binary vector T-DNA, but no Ri-plasmid T-DNA. Theor. Appl. Genet. 72: 770–777.

Sharma, M.K., Solanke, A.U., Jani, D., Singh, Y., and Sharma, A.K. 2009. A simple and efficient *Agrobacterium*-mediated procedure for transformation of tomato. J. Biosci. 34: 423–433.

Subramanyam, K., Subramanyam, K., Sailaja, K.V., Srinivasulu, M., and Lakshmidevi, K. 2011. Highly efficient *Agrobacterium*-mediated transformation of banana cv. Rasthali (AAB) via sonication and vacuum infiltration. Plant Cell Rep. 30(3): 425–436.

Suganuma, H., Hirano, T., Kaburagi, S., Hayakawa, K., and Inakuma, T. 2004. Ameliorative effects of dietary carotenoids on memory deficits in senescence-accelerated mice (SAMP8). Int. Congr. Ser. 1260: 129–135.

Sun, H.J., Uchii, S., Watanabe, S., and Ezura, H. 2006. A highly efficient transformation protocol for Micro-Tom, a model cultivar for tomato functional genomics. Plant Cell Physiol. 47: 426–431.

Tabei, Y., Yamanaka, H., and Kanno, T. 1993. Adventitious shoot induction and plant regeneration from cotyledons of mature seed in watermelon (*Citrullus lanatus L.*). Plant Tiss Cult Lett. 10: 235–241.

Van Roekel, J., Damm, B., Melchers, L., and Hoekema, A. 1993. Factors influencing transformation frequency of tomato (*Lycopersicon esculentum*). Plant Cell Rep. 12: 644–647.

Vernade, D., Herrera-Estrella, A., Wang, K., and Van Montagu, M. 1988. Glycine betaine allows enhanced induction of the *Agrobacterium tumefaciens* vir genes by acetosyringone at low pH. J. Bacteriol. 170: 5822–5829.

Vidya, C.S.S., Manoharan, M., Kumar, C.T.R., Savithri, H.S. and Sita, G.L. 2000. *Agrobacterium*-mediated transformation of tomato (*Lycopersicon esculentum* var. Pusa Ruby) with coat-protein gene of Physalis mottle tymovirus. J. Plant Physiol. 156: 106–110.

Wangxia, W., Vinocur, B., and Altman, A. 2003. Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta. 218: 1–14.

Wu, Y.F., Chen, Y., Liang, X.M., and Wang, X.Z. 2006. An experimental assessment of the factors influencing *Agrobacterium*-mediated transformation in tomato. Russ. J.Plant Physiol. 53: 252–256.

Yeung, E.C., Aitken, J., Biondi, S., and Thorpe, T.A. 1981. Shoot histogenesis in cotyledon explants of radiata pine. Botan. Gaz. 142: 494–501.

Ying, C.Y., Huang, X.Q., Guo, Y.Q., Zhong, L.L., Liu, Y., Li, S.L., Gu, X.M., and Zhou, X.H. 2008. Optimization of tomato genetic transformation, kanamycin-resistant screening and seed selection. J. South. Med. Univ. 28: 1117–1122.

Zhang, H.X., and Blumwald, E. 2001. Transgenic salt-tolerant tomato plants accumulate salt in foliage but not in fruit. Nat. Biotechnol. 19: 765–768.

****

How to cite this article:

Rajesh N *et al*. 2016, An Establishment of Efficient Agrobacterium Mediated Transformation In Tomato (Solanum Lycopersicum). *Int J Recent Sci Res.* 7(1), pp. 8583-8591.

TSSN 0976-3031