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Full Length Article

Enhanced Somatic Embryogenesis and *Agrobacterium*-Mediated Transformation of three Cultivars of Tomato by Exogenous Application of Putrescine

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Abstract

Efficiency of *Agrobacterium* mediated transformation of tomato with coat protein (CP) of *Tomato yellow leaf curl virus* (TYLCV-OM) was optimized using different media. Cotyledons and epicotyls parts from susceptible tomato cvs. Moneymaker, Pusa Ruby and Jinan were used as explants and co-cultivated with *Agrobacterium tumefaciens* strain AGL1 harbouring CP gene. The effects of putrescine, vitamins, explants and growth regulators on plant transformation and regeneration were studied. Optimal shoot regeneration and callus induction was obtained with 1 µM putrescine, 0.5 µg/mL zeatin riboside and 0.1 µg/mL indole acetic acid. Maximum transformation efficiency was obtained with bacterial concentration of $1x10^8$ cells incubated for 48 h. The above conditions increased transformation efficiency up to 49.2% using cotyledons as explant. Gene specific primers were used to confirm the presence of transgene in the primary transformants selected on kanamycin. Transformation efficiency using cotyledons and epicotyl was found to be 49.2%, 33% in Moneymaker, 32%, 27% Jinan and 48.1%, 32% in Pusa Ruby respectively. All the values were found significantly different ($p< 0.05$) using LSD. The study shows that exogenous application of putrescine combined with zeatin riboside can enhance regeneration ability of cotyledon as well as epicotyl and decrease rhizogenesis and expansion of necrotic lesions. © 2014 Friends Science Publishers

Keywords: Tomato; Somatic embryogenesis; Transformation efficiency; Putrescine; Zeatin riboside

Introduction

Belonging to *Solanaceae* family, tomato (*Solanum lycopersicum* L.) is one of the important crops worldwide with crop production over 141 million tons over an area of 5 million hectares (Mueller *et al*., 2005). In Oman, tomato production reached 81000 tons (FAOSTAT, 2010. The enormous number of available researches working on tomato gives way to the development of new processes and tools for genomics and genetic analyses (Barone *et al*., 2008). The major focus in tomato biotechnology has been the introduction of foreign gene(s) into tomato genome for the enhancement of tomato production by increasing the tendency to fight against biotic and or abiotic stresses (Arumuganathan and Earle, 1991). Researchers have employed several techniques such as particle bombardment, microinjection, *Agrobacterium* mediated transformation and protoplast fusion to introduce foreign genes into plant genome (Otoni *et al*., 2003). Use of *Agrobacterium* mediated transformation has been the method of choice due to high transformation rate and the ability of *Agrobacterium* to integrate foreign genes into plant genome naturally (Otoni *et al*., 2003). The *Agrobacterium* mediated plant transformation was first reported by McCormick *et al.* (1986) which followed many publications on engineering of tomato to confer resistance against environmental stresses and to provide nutritionally improved transgenics (Janssen *et al.*, 1998; Park *et al*., 2003; Lin *et al.*, 2004; Davuluri *et al*., 2005; Youm *et al.*, 2008).

Transformation efficiency is influenced by several factors, which include explants (cotyledons, stem and leaf), plant variety, growth regulators and bacterial concentration (Cortina and Culiáñez-Macià, 2004). Tomato transformation efficiency reported in literature, so far, is 9% (Roekel *et al*., 1993), 11% (Frary and Earle, 1996), 20% (Qiu *et al*., 2007), 41.4% (Sharma *et al*., 2009) and 28‑48% (Sun *et al*., 2006). The development of improved and genotype independent transformation procedure is crucial for successful results. Cotyledonary leaves are the best choice for clonal propagation (Ellul *et al*., 2003). However, unlike juvenile tissues, mature tissues (stem, epicotyl and hypocotyl) have been reported with reduced morphogenic capacity and recalcitrant *in vitro* behavior (Diaz-Sala *et al*., 1990a; Yu and Reed, 1995). Insufficient shoot elongation, low rates of multiplication and callus initiation, higher rates of microbial contamination are the main factors reducing the success of

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explants transformation (Diaz-Sala *et al*., 1990a; Yu and Reed, 1995).

Endogenously produced spermine (Spm), putrescine (Put) and spermidine (Spd) are the major plant polyamines which have been widely documented to play significant role in cell development (Minocha and Minocha, 1995). Increased level of polyamine Put has been shown to correlate with enhanced somatic embryogenesis and transformation in several plant species (Kakkar and Sawhney, 2002; Kevers *et al*., 2002; Sakhanokho *et al*., 2005). Higher level of free Put in juvenile tissues has been associated with the ease of culture establishment (Diaz-Sala *et al*., 1990b; Bais *et al.*, 2002). Moreover, it has been reported that exogenous application of Put may reduce the production of unwanted ethylene and enhance morphogenesis, which in turn increases the explant survival and transformation efficiency (Bais *et al*., 2000).

The present study was conducted to develop a reproducible, simplified and genotype independent tissue culture protocol with enhanced transformation efficiency by avoiding feeder layer in tomato cultivars. Callus induction and regeneration was optimized in susceptible tomato cultivars using specific combination of growth regulators supplemented with Put. The study reports the selection and optimization of plant growth regulators and Put to enhance *Agrobacterium* mediated transformation of CP gene of TYLCV-OM in Moneymaker, Jinan and Pusa Ruby cultivars of tomato using cotyledons and epicotyls as explant.

Materials and Methods

Seed Sterilization

Cotyledons and epicotyls from 9 days old tomato cvs. Moneymaker, Jinan and Pusa Ruby susceptible to TYLCV-Om, were used as explants. Seeds (100 each) of three tomato cultivars were washed 3 times with sterilized distilled water followed by dipping in 13% liquid bleach (Clorox) for 2-5 min. Seeds were washed again for 6 times with sterilized distilled water to remove excess bleach. Germination of surface sterilized seeds was achieved (Table 1) in growth chamber under fluorescent light with 12 h photoperiod. For preparation of explants, cotyledons and epicotyls from germinated seedlings were used.

Culture Media

Murashige and Skoog (1962) basal medium supplemented with different chemicals was used in this study (Table 1). Before adding 0.8% plant tissue-culture grade phyto agar powder, the media components were adjusted to pH of 5.8 (Duchefa Biochemie BV, Netherlands) and autoclaved. After cooling the autoclaved medium to ~50°C, growth regulators and filter sterilized antibiotics were added.

Bacterial Strain and Plasmids

Agrobacterium tumefaciens strain AGL1 was utilized for plant transformation. Binary vector pGreen0029 (Hellens *et al*., 2000) containing promoter pMAS (monoamine synthase promoter) and terminator tMAS (monoamine synthase terminator) cloned at *Eco*RV site was used for transformation. A595bp fragment of coat protein gene of TYLCV-OM was synthesized and subcloned into pUC57 vector (GenScript Inc., NJ, USA). The whole gene was lifted from pUC57 using *Sal*I/*Eco*RI and cloned into pGreen0029 vector at the same site. The clones were confirmed by restriction digestion using *Sal*I/*Eco*RI site (Fig. 1). The vector was then electroporated in *A. tumefaciens* AGL1 strain and colony PCR was performed to identify the transformants.

To test the optimal bacterial concentration required for successful transformation, four bacterial densities 0.5 $x10^8$, 0.6 $x10^8$, 0.8 $x10^8$ and 1.0$x10^8$cells/mL were used for co-cultivation of explants for 48 and 72 h (Table 2). Single colony from freshly grown plate was picked up and inoculated in 10 mL YEM (yeast extract mannitol) medium containing 50 mg/L kanamycin and 100 mg/L Carbenicillin. It was incubated at 28°C for 48 h with continuous shaking at 200 rpm. Five mL of bacterial culture at OD_{600} 0.8-1.0 was used to inoculate 50 mL of fresh YEM medium and shaked for 3-4 h at 200 rpm until it reached OD_{600} 0.5-0.8. At this point 200 µM Acetosyringone was added and incubated for l h. *Agrobacterium* cells were centrifuged at 6000 rpm for 10 min and re-suspended in 50 mL fresh YEM medium and used for transformation.

Optimization of Growth Regulators on Tomato Explants Survival

Significant effects were noticed on different hormone combination on shoot elongation and regeneration medium. Three different concentrations of zeatin (Z) (0.5, 1 and 1.5) µg/mL, zeatin-riboside (ZR) (0.5, 1 and 1.5) µg/mL, 6-benzylaminopurine (BAP) (1, 1.5 and 2) µg/mL, 1-naphthaleneacetic acid (NAA) (1, 1.5 and 2) µg/mL and indole acetic acid (IAA) (0.1, 0.4 and 1) µg/mL were selected to enhance transformation efficiency. To study the effect of Put, four concentrations (0.1, 1, 1.5 and 2 mM) were used in combination with other hormones. All concentrations were tested in control conditions with 100 explants in each petri dish with three replicates of each treatment. The combination of growth regulators and

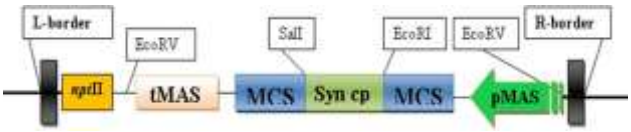

Fig. 1: Schematic presentation of T-DNA border of plant transformation vector pGreen0029 containing CP gene under monoamine synthase promoter/terminator

polyamine that gave highest regeneration efficiency was selected to make regeneration media. Different combinations of plant growth regulators with or without Put in MS basal medium (Table 3) were used to improve tomato transformation efficiency with some modification reported previously (Rogozinska and Skutnik, 1974; Cortina and Culiáñez-Macià, 2004; Raj *et al*., 2005; Sharma *et al*., 2009). M1 is a combination of Z and IAA, whereas in M2 same media was supplemented with Put to see the role of polyamine in transformation efficiency. In M3 combination Z is replaced with ZR to test trans isomer with IAA and in M4 same media was supplemented with Put. In M5 ZR was combined with NAA and BAP and in M6 Put was added in same combination (Table 3). All experiments were repeated three times using 100 explants each time. Regenerated plantlets from single callus after transformation were labeled as clones.

Plant Transformation

Nine days old seedlings were used to cut cotyledons at the base and tip. Middle pieces (~0.5 cm × 0.7 cm) were incubated with the adaxial surface in contact with the pre-culture medium at 28°C for 48 h (Table 1). Explants, which showed signs of swelling meaning positive response to pre-culture were incubated under bacterial suspension for 30 min and were flipped upside down every 10 min during the incubation process. The explants were then blotted dry and co-cultured on the same pre-culture medium in the dark for 48 h at 28°C. For regeneration of co-cultured explants, they were transferred to selection medium (Table 1). Almost 20–25 explants for regeneration were present in each petri plate (9 cm). The plates were cultured at 28°C under 16 h light/8 h dark cycle. Using fresh selection medium every 15 days the explants showing regeneration or callus formation were sub-cultured. The regenerated shoots having callus were transferred to shoot elongation medium (Table 1). The shoots were excised from callus and transferred to rooting medium (Table 1). Plantlets, which developed shoot (~8 cm in height) and also produced plentiful roots were shifted to pots containing sterilized potting soil for hardening. These pots were then kept for 5 days in humidity chamber under 28°C and 16 h light/8 h dark cycle after that moved to glasshouse.

All data obtained from regeneration and transformation using different explants and plant growth regulators were statistically analyzed using ANOVA. Using least significant difference test (LSD) at $P < 0.05$ means were compared.

Transgene Confirmation

Plant genomic DNA was extracted using 0.1 g fresh leaf tissues as described by Doyle and Doyle (1990). The total nucleic acid extracted from transgenic tomato leaves was used as template to amplify CP gene using primer pair (Forward: 5'-TCAAAGAGGCCAGGGGACAT-3' / Reverse: 5'- GCGTGTGTGCAAGCCATGTA-3'). The PCR amplification was carried out using 25 µL reaction mix (10X Thermo Pol reaction buffer, 0.5 µL of 10 mM dNTPs, 0.2 µL Taq DNA polymerase, 1 µL of 3 mM of $MgCl_2$, 18.8 µL sterile distilled water, 0.5 µL of 10 µM of each primer, and 1 µL of template DNA) using Express thermo cycler (Hybaid Ltd., Middlesex, UK). Initial denaturation was done for 1 min at 95°C followed by 35 cycles of denaturation at 96°C for 45 s, annealing at 53°C for 1 min and extension at 75°C for 2 min with final extension at 75°C for 5 min. The amplified DNA fragments were electrophoresed through 1.4% (w/v) agarose gel in 0.5X TAE buffer stained with ethidium bromide solution (0.5 µg/mL). The DNA bands were visualized under UV light and photographed using Syngene gel documentation system. Size of the amplicons was estimated in comparison with 100 bp DNA ladder.

Results

Begomovirus susceptible tomato varieties Moneymaker, Pusa Ruby and Jinan were used in this experiment to optimize somatic embryogenesis and CP gene transformation. Cotyledons and epicotyls from 9-days-old seedlings were used as explants and cultured on 1X MS pre-culture medium supplemented with 1 mM Putrescine, 1 mg/L zeatin riboside, and 0.1 mg/L IAA (Table 1). Cotyledons and epicotyls were cut at both sides and the middle portion was used as explant by placing their adaxial side touching medium. More than 90% of the explants responded to the pre-culture conditions by expanding in size within 48 h.

Consequences of Bacterial Concentration on Transformation

The optimum concentration of bacterial culture needed for successful transmission of CP gene was found to be 1.0×10^8 cells/mL co-cultivated for 48 h (Table 2). Bacterial density of 0.5×10^8 cells/mL with 48 h co-cultivation time gave 28.6% transformation efficiency, which increased to 32.2% after 72 h (Table 2). Bacterial concentration 0.8×10^8 cells/mL with co-cultivation time of 48 h yielded 45.7% transformants (Table 2). Bacterial concentrations of 1.0×10^8 and 0.8×10^8 cells/mL with co-cultivation time of 48 h were found to be the most efficient showing 49.2% and 45.7% transformation, respectively. However, co-cultivation for 72 h decreased the transformation by 40-51% for both bacterial concentrations (Table 2). Additionally, excessive growth of *Agrobacterium* was evident when a co-cultivation time 72 h used, which could have affected the overall survival of explants.

Optimization of Various Factors Affecting Tomato Regeneration and Transformation

In control experiments, 0.5 µg/mL zeatin or zeatin riboside, 1 µg/mL BAP, 1 µg/mL NAA and 0.1 µg/mL IAA concentrations were found to enhance shoot

Table 1: Concentrations of different growth regulators used in various transformation and regeneration media

	Seed germination	Pre-culture	Co-culture	Selection/ shooting[a]	Shoot elongation	Rooting
Putrescine (mM)		1	1	1	1	1
BAP (mg/L)					0.5	
Zeatin Riboside (mg/L)		1	1	0.5		
IBA (mg/ml)						1
IAA (mg/ml)		0.1	0.1	0.05		
Kanamycin (mg/ml)				50	25	50
Cefotaxime (mg/ml)				250	250	-
MS[b]	1x	1x	1x	1x	0.5x	0.5x
Sucrose (g/L)	30	30	30	30	30	30
Phytoagar (g/L)	8	8	8	8	8	8
Gamborg vitamins[c]	0.5x	1x	1x	1x	1x	1x
Acetosyringone (mM)			200			
pH	6.0	5.8	5.8	5.8	5.8	5.8

[a]Different combinations of hormones were used in this medium. For details see Table 3; [b]Murashige and Skoog (1962); [c]Gamborg *et al.* (1968)

Table 2: Effect of bacterial concentration and incubation time on overall efficiency of tomato transformation

Bacterial OD (cells/ml)	Transformation efficiency (%) ± SE	
	48 h Co-cultivation	72 h Co-cultivation
0.5×10^8	28.6 ± 1.5	32.2 ± 2.8
0.6×10^8	30.2 ± 1.3	33.4 ± 1.5
0.8×10^8	45.7 ± 2.3	27.8 ± 2.5
1×10^8	49.2 ± 2.8	24. 4 ± 2.1

Table 3: Different growth regulator recipes with and without putrescine on three tomato cultivars regeneration/shoot development stages

Media no.	MS medium with different growth regulators (mg/L)	Regeneration efficiency in control					
		Moneymaker		Pusa ruby		Jinan	
		Cotyledons	Epicotyl	Cotyledons	Epicotyl	Cotyledons	Epicotyl
M1	Z (0.5) + IAA (0.1)	47.7±2.1	28.7±1.5	42.0±2.0	23.0±2.0	43.3±2.1	24.0±2.0
M2	Z (0.5) + IAA (0.1) + Put (1mM)	53.3±1.5	43.0±2.0	47.3±1.5	37.0±2.0	42.0±1.0	30.7±3.5
M3	ZR(0.5)+ IAA (0.1)	58.0±1.7	36.3±1.5	52.0±1.7	30.3±1.5	51.0±2.0	27.7±3.1
M4	ZR(0.5)+IAA(0.1)+Put (1mM)	92.3±2.1	44.7±1.2	86.3±2.1	38.7±1.2	79.0±3.0	43.0±2.0
M5	ZR(0.5)+NAA(1)+BAP (1)	10.7±2.1	4.7±1.2	8.7±2.1	2.7±1.2	6.0±1.7	2.0±1.0
M6	ZR(0.5)+NAA(1)+BAP(1)+Put(1mM)	16.0±3.6	8.0±1.7	14.0±3.6	6.0±1.7	12.0±1.0	5.0±2.0

regeneration/elongation. The number of shoots per explants was increased with the addition of 1 mM of Put. Six different combinations of growth regulators labeled as M1-M6 (Table 3) were tested in non co-cultivated control experiment using Moneymaker, Pusa Ruby and Jinan varieties as explants. All combinations (M1-M6) showed positive response in control experiments that were further used to test their individual effect on transformation efficiency (Table 3). MS medium with M1 combination showed highest regeneration efficiency in Moneymaker with cotyledon (47.7%) and epicotyl (28.7%) as explants (Table 3). M1 combination supplemented with 1 mM Put (M2) showed increasing trend of transformation for all varieties. In M3 combination, zeatin was replaced by its trans isomer zeatin riboside, which showed positive effect on cotyledon explants (Table 3). Epicotyl explants responded well in M2 than M3 combination (Table 3). The addition of Put in M4 combination further improved regeneration in both explants for all three cultivars (Table 3). M4 combination resulted highest regeneration efficiency of 92.3% and 44.7% in cotyledon and epicotyl, respectively (Table 3). Poor regeneration response was noticed with zeatin riboside + NAA + BAP (M5). Addition of 1 mM Put to M5 combination increased the regeneration efficiency (M6 combination). Tomato variety Moneymaker was found to produce highest regeneration on all media combination followed by Pusa Ruby and Jinan. In case of explants, cotyledons and epicotyl showed variable response to regeneration on different media combination (Table 3). For all tomato varieties tested, cotyledon explants responded higher for regeneration as compared to epicotyls. Media combination M4 with highest regeneration efficiency of 92.3 % in Moneymaker was selected to make optimized method for transformation as presented in Table 1.

Effects of different hormone combinations on transformation efficiency are shown in (Fig. 2a and b). In M1 combination (Fig. 2a) highest transformation efficiency was observed in Moneymaker 12% and 10% with cotyledons and epicotyls respectively (Fig. 3a and b). In M2 combination, addition of 1 mM Put showed an increase in shoot regeneration even after two weeks of co-cultivation (Fig. 2b). Comparable results were obtained in cotyledon

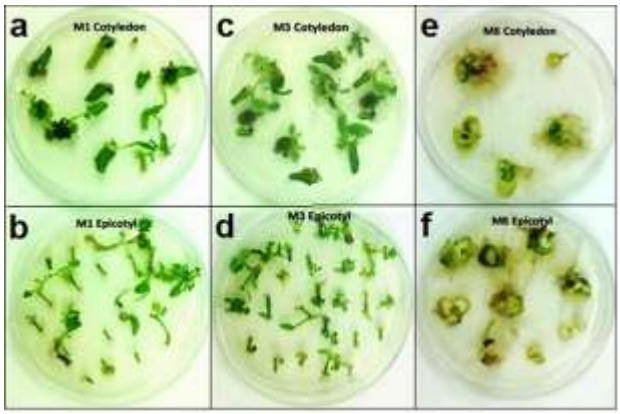

Fig. 2a: Effect of various growth regulators combinations without putrescine on callus initiation and shoot regeneration medium after two weeks of cocultivation. (a) Cotyledonary explants cultured on M1 (Zeatin + IAA) medium (b) Epicotyl explants cultured on M1 (zeatin + IAA) medium (c) Cotyledonary explants cultured on M3 (zeatin riboside + IAA) medium (d) Epicotyl explants cultured on M3 (zeatin riboside + IAA) medium (e) Cotyledonary explants cultured on M5 (zeatin riboside + NAA + BAP) medium (f) Epicotyl explants cultured on M5 (zeatin riboside + NAA + BAP) medium

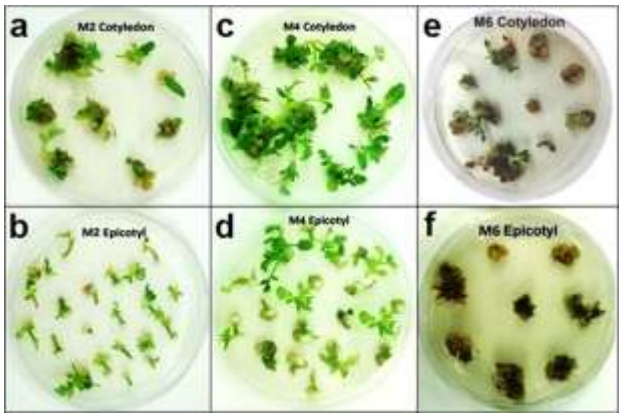

Fig. 2b: Effect of various growth regulators combinations with putrescine on callus initiation and shoot regeneration medium after two weeks of cocultivation. (a) Cotyledonary explants cultured on M2 (zeatin + IAA + Put) medium (b) Epicotyl explants cultured on M2 (zeatin + IAA+ Put) medium (c) Cotyledonary explants cultured on M4 (zeatinriboside + IAA+ Put) medium (d) Epicotyl explants cultured on M4 (zeatinriboside + IAA+ Put) medium (e) Cotyledonary explants cultured on M6 (zeatinriboside + NAA + BAP + Put) medium (f) Epicotyl explants cultured on M6 (zeatinriboside + NAA + BAP + Put) medium

explants of Pusa Ruby 24.6% and Moneymaker 25.7% cultivar (Fig. 3a) using M2 combination. In M3 media trans isomer zeatin riboside was used instead of zeatin. M3 combination showed 17% transformation efficiency with cotyledon and 16% with epicotyls in Moneymaker (Fig. 3a and b). Insufficient number of shoots was observed in M1 and M3 medium which rendered them useless for any further use. M3 media supplemented with 1 mM Put in M4 combination showed significant increase in transformation efficiency in all three cultivars (Fig. 3a and b). Maximum shoots per explants were detected in M4 medium even after two weeks of co-cultivation and their survival rate was high enough to turn into transgenic plants. In M4 combination of growth regulators highest transformation efficiency was observed in Moneymaker i.e., 49.2% and 33% in cotyledons and epicotyl explants respectively (Fig. 3a and b). Whereas in M2 combination, zeatin instead of zeatin riboside along with 1 mM Put showed less regeneration efficiency (25.6% in cotyledons and 24.3% in epicotyls (Fig. 3a and b).

The browning of callus and rhizogenesis was observed on M5 media indicating their negative effects on tomato tissue culture that needed to be further improved. Callus observed turned to brown color and shoot tips showed necrosis by second sub-culturing period. In M6 combination, where Put was added to enhance transformation efficiency, a slight increase in transformation efficiency was observed (Fig. 3a and b).

According to current observation zeatin riboside combined with 1 mM Put proved to enhance regeneration ability of mature as well as immature tissues (Fig. 2b, 3a and 3b). When regenerated shoots were moved to proliferation medium with zeatin riboside (0.5 µg/mL), IAA (0.1 µg/mL) and 1 mM Put (Fig. 2b), highest number of shoots were observed per explant. Without growth regulators in MS basal medium there was no regeneration response in explants.

Plant Transformation

The earliest responses of explants to different *in vitro* conditions included leaf expansion, callus induction and shoot initiation. Basal medium M3 was used to culture CP gene transformants. Kanamycin resistant green calli with initiated shoots were observed after 2 to 3 weeks post transformation (Fig. 4c). Cotyledon explants were removed from the regenerated shoots after 4-8 weeks of shoot initiation. Medium supplemented with 1 mM Put, 1.0 mg/L BAP, 50 mg/L kanamycin and 250 mg/L cefotaxime showed enhanced shoot initiation and elongation after 8 weeks post inoculation. Half strength MS-B5 medium supplemented with IAA (0.1 µg/mL), 1 mM Put and kanamycin (50 mg/L) was found suitable for rooting of transformed shoots, where 92% shoots produced roots after 10 to 12 days.

Strong hairy roots were formed after 10 days when regenerated plantlets with some initial roots were transferred to half-strength liquid MS-B5 medium supplemented with kanamycin and cefotaxime (Fig. 4 g). The transformed plants with strong rooting system were transferred to small pots where 90% survival rate was witnessed. Completely regenerated transgenic plants were transferred to phytotron. The acclimatized transgenic plants grew well, until maturity and produced normal fruits (Fig. 4i and j). All plants were found morphologically normal and fertile.

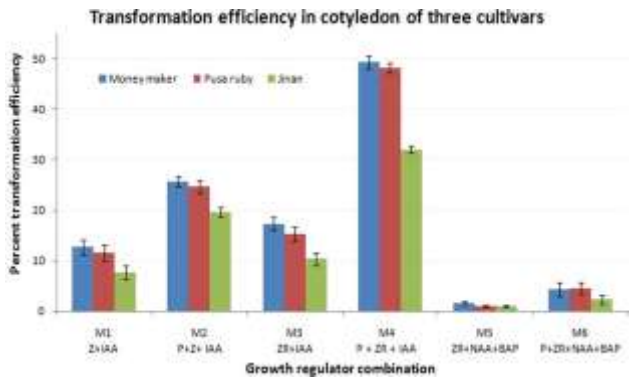

Fig. 3a: Effect of growth regulators combination on shoot regeneration medium on Moneymaker, Pusa Ruby and Jinan cultivar. Higher percentage of explants survival (cotyledon) was observed with 1mM putrescine and 0.5 mg/ml zeatin riboside M3. Data represents the mean value of six independent experiment (100 explants for each independent transformation procedure) ±SD. Bars represent standard errors. All the values were found to be significantly different ($p< 0.05$) using LSD

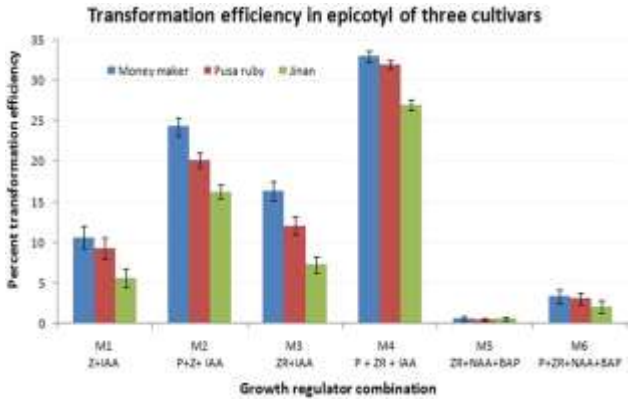

Fig. 3b: Effect of different combinations of growth regulators on shoot regeneration medium on Moneymaker, Pusa Ruby and Jinan cultivar. Higher percentage of explants survival (epicotyl) was observed with 1mM putrescine and 0.5mg/ml zeatin riboside M3. Data represents the mean value of six independent experiment (100 explants for each independent transformation procedure) ± SD. Bars represent standard errors. All the values were found to be significantly different ($p< 0.05$) using LSD

Molecular Examination of T0 Transgenic Plants

DNA from a non-transformed control and five independently transformed - T0 transgenic lines (L1-L5) was extracted using CTAB method. A healthy tomato plant was used as a wild type control and the CP gene cloned in pGreen 0029 vector was used as positive. DNA was PCR amplified using primers specific for CP gene (Fig. 5). A 595 bp DNA band corresponding to the predicted size of gene fragment confirmed T-DNA integration in the genome of these plants.

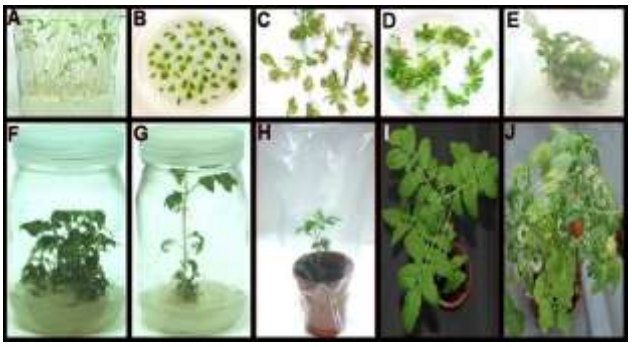

Fig. 4: Different stages of *Solanum lycopersicum* var. Moneymaker transformation. (a) Germinated seedlings on seed germination medium. (b) Cotyledonary explants cultured on pre-culture medium. (c) Cotyledonary explants on selection medium after 2 weeks of co-cultivation with *Agrobacterium* harbouring CP gene (d) Callus with regenerating shoots growing on selection medium (e) Multiple shoot bud formation from single explants. (f) Multiple shoots regenerating from a single callus. (g) Young plantlet with well-developed rooting system on selection medium (h, i) Soil acclimated plant **(j)** Mature transgenic tomato plants bearing fruit

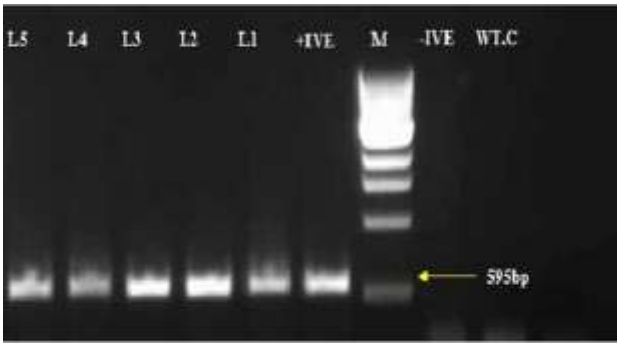

Fig. 5: PCR mediated detection of transgene (CP) in transgenic tomato plants. The ethidium bromide-stained agarose gel was photographed under UV illumination. The samples loaded on the gel resulted from PCR reactions with primer pair (CP1/CP2) and DNA extracted from the leaves of tissue culture prepared tomato plants. The band size of 595 bp indicates the presence of transgene. Samples loaded on the gel are L1, L2, L3, L4 and L5. A DNA size marker was electrophoresed in lane 3

Discussion

Transformation efficiency in tomato depends on several factors such as explants type (cotyledons, stem and leaf), cultivar and age of explants, co-cultivation time, *Agrobacterium* strain and cell density and type of regeneration medium (Davis *et al*., 1991). Cotyledon explants are well established to get good regeneration in tomato (Frary and Earle, 1996; Ellul *et al*., 2003). After 48 h incubation of explants (cotyledons and epicotyls) on pre-culture medium, we found more than 90% of explants responded positively. Though this response was not similar in all explants, only those explants were selected for co-

cultivation which responded to pre-culture medium. Previous reports suggested that tomato transformation process becomes tedious when feeder layer was used during pre-culture/co-culture medium (McCormick *et al*., 1986; Cortina and Culiáñez-Macià, 2004; Frary and Van Eck, 2005). Current studies of pre-culture/co-cultivation medium contained zeatin riboside (0.5 µg/mL) combined with 1mM Put without using feeder layer similar to that reported by Qiu *et al.* (2007).

The optimum concentration of bacterial culture needed for successful transmission of cp gene (Table 2) was found to be 1.0x 10^8cells/mL for 48 h, being inconsistent with the results of Sharma *et al.* (2009). We witnessed higher transformation efficiency (32% for Jinan, 49.2% for Moneymaker and 48.1% for Pusa Ruby) in comparison to 14.2% and 41.4% reported earlier by Raj *et al.* (2005) and Sharma *et al*. (2009), respectively. The increased transformation efficiency may be due to the collective effect of bacterial concentration, plant growth regulator combination and co-cultivation time. When the bacterial concentration was increased from 1.0 × 10^8 cells/mL or increased co-cultivation time from 48 h, it adversely affected the explants and the recovered turned brown and subsequently died (data not presented). The explants turned soft and black and subsequently lost the regeneration capability. Substantial reduction in transformation efficiency was seen when co-cultivated for 72 h at bacterial density 1.0 × 10^8 cells/mL. Furthermore, increase of incubation time to 96 h significantly decreased explants regeneration (data not shown). The results show that for successful plan transformation there is a threshold limit for bacterial OD and time of co-cultivation.

Murashige and Skoog (1962) medium is the most frequently used medium in tissue culture. However, a high percentage of tomato cotyledons explant developed necrosis when cultured on MS medium. The increase in thiamine concentration in MS medium allowed cell growth and decreased the expansion of necrotic lesions. Gamborg vitamins by contains ten-fold higher concentration of thiamine HCl as compared to MS which has pronounced effect on overall cell-growth/proliferation in transformed tissues (Gamborg *et al.*, 1968). We used MS medium supplemented with Gamborg vitamins to enhance overall transformation and regeneration efficiency of putative explants (Table 1). Similar results were found by Raj *et al.* (2005). Based on previous modifications of MS medium, M1 combination (Table 3), Fig. 2a showed green calli but the overall efficiency was found low in contrast to Cortina and Culiáñez-Macià (2004).

Transformation efficiency obtained in M3 (ZR+ IAA) media was superior to that observed in M1 (Z+ IAA) combination having trans isomer ZR. As previously reported, Z combined with IAA improves callus initiation and shoots regeneration (Park *et al.,* 2003). In M3 medium, trans isomer of Z was used in combination with IAA. Enhanced transformation efficiency has also been observed using trans-isomer of Z (ZR) by Cortina and Culiáñez-Macià (2004).

Exogenous application of Put can decrease the production of undesirable ethylene and increases morphogenesis which in turn raises the explants' survival and percent transformation efficiency (Minocha and Minocha, 1995; Bais *et al*., 2000; Kakkar and Sawhney, 2002; Kevers *et al*., 2002; Sakhanokho *et al*., 2005). In the current observation M2 and M4 combinations of growth regulator were made using Z and trans isomer ZR in combination with Put and IAA. The inclusion of Put in M4 media was found superior to M2 combination. M2 combination showed less regeneration efficiency whereas explants on M4 media (Fig. 3a and b) showed increased shoot regeneration even after two weeks of co-cultivation (Fig. 2b). In our study, M4 medium showed highest transformation efficiency with cotyledons and epicotyl 49.2%, 33% in Moneymaker, 32%, 27% Jinan and 48.1%, 32% in Pusa Ruby respectively. Highest regeneration efficiency of cotyledon explants 92.3% in Moneymaker, 86.3% in Pusa Ruby and 79% in Jinan was observed (Table 3). All the varieties responded positively to M4 combination both in control and transformation experiments with both explants (Fig. 3a and 3b). Highest Transformation efficiency 49.2% in cotyledons and 33% in epicotyl explants (Fig. 3a and 3b) was observed in M4 combination. The improved transformation efficiency observed using Put in the regeneration medium was statistically significant at $p< 0.05$.

Rogozinska and Skutnik (1974), used combination of BAP and NAA for shoot regeneration in tomato showing superior effects. Park *et al*. (2001) and Sun *et al.* (2006), also reported same medium for regeneration. However, in the present investigation when explants were cultured on M5 and M6 growth regulator combinations rhizogenesis was observed (Fig. 2a and b). It was observed that addition of Put slightly increased the transformation efficiency in M6 combination. The number of survived transformed explants observed in M6 combination was not high enough to get transgenic lines.

Most of transgenic plants showed CP gene integration. Transgene confirmation was done by PCR by using total genomic DNA isolated from leaves of transgenic tomato plants. The transformation efficiency was improved significantly for Pusa ruby (48.1%) compared with reported earlier (Raj *et al*., 2005; Sharma *et al*., 2009). The media combinations were also effective in transforming tomato cultivars Moneymaker and Jinan (Omani). The improved protocol is simple, reproducible, and could be used for other tomato varieties.

In conclusion, addition of 1 mM putrescine along with 0.5 µg/mL zeatin riboside and 0.1 µg/mL IAA (M4 media) in MS, increased cell growth and decreased the expansion of necrotic lesions. Using cytokinin zeatin riboside, instead of zeatin, increased shoot regeneration rate. Cotyledons showed highest regeneration and transformation efficiency with tomato cultivars, Moneymaker 49.2%, Jinan 32% and Pusa Ruby 48.1%, respectively. Transformation efficiency was

improved four times and 94% of the resistant shoots rooted on kanamycin and confirmed as transgenic plants. The results presented here indicate that addition of polyamines (putrescine) in the culture medium could enhance the morphogenic capacity of explants for the establishment of transgenic tomatoes. This is simplified and improved tomato regeneration protocol to produce transformed plants through *Agrobacterium*-mediated transformation.

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References

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

Bais, H.P. and G.A. Ravishankar, 2002. Role of polyamines in the ontogeny of plants and their biotechnological applications. *Plant Cell Tiss. Org. Cult*., 69: 1–34

Bais, H.P., G. Sudha and G.A. Ravishankar, 2000. Put and silver nitrate influence shoot multiplication, *in vitro* flowering and endogenous titers of polyamines in *Cichoriumintybus L.* cv. *Lucknow local. J. Plant Growth Regul*., 19: 238–248

Barone, A., M.L. Chiusano, M.R. Ercolano, G. Giuliano, S. Grandillo and L. Frusciante, 2008. Structural and functional genomics of tomato. *Int. J. Plant Genom.* doi., 10.1155/2008/820274

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

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

Davuluri, G.R., T.A. Van, P.D. Fraser, A. Manfredonia, R. Newman, D. Burgess, D.A. Brummell, S.R. King, J. Palys, J. Jhlig, P.M. Bramley, H.M.J. Pennings and C. Bowler, 2005. Fruit-specific RNAi-mediated suppression of *DET1* enhances carotenoid and flavonoid content in tomatoes. *Nat. Biotechnol*., 23: 890–895

Diaz-Sala, C., M. Rey and R. Rodriguez, 1990a. In Vitro establishment of cycloclonal chain from nodal segments and apical buds of adult hazel (*Corylusavellana* L.). *Plant Cell Tiss. Org. Cult*., 23: 151–157

Diaz-Sala, C., M. Rey and R. Rodriguez, 1990b. Recovery of transient juvenile apacities during micropropagation of filbert. *In*: *Plant Ageing: Basic and Applied Approaches*, pp: 27–36. Rodriguez, R., T.R. Sanchez and D.J. Durzan, (eds.). NATO, ASI Series, Series A, Life Sciences, Plenum Press, New York

Doyle, J.J. and J.L. Doyle, 1990. Isolation of plant DNA from fresh tissue. *Focus*, 12: 13–15

Ellul, P., B. Garcia-Sogo, B. Pineda, G. Rios, L.A. Roig and V. Moreno, 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

FAOSTAT, 2010; http: //faostat.org. Accessed: 20 February 2013

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

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

Gamborg, O.L., R.A Miller and K. Ojima, 1968. Nutrient requirements of suspension culture of soybean root cells. *Exp. Cell Res*., 50: 151–158

Hellens, R.P., E.A. Edwards, N.R. Leyland S. Bean and P.M. Mullineaux, 2000. pGreen: a versatile and flexible binary Ti vector for *Agrobacterium* mediated plant transformation. *Plant Mol. Biol.*, 42: 819–832

Janssen, B., L. Lund and N. Sinha, 1998. Overexpression of a homeobox gene, *LeT6*, reveals indeterminate features in the tomato compound leaf. *Plant Physiol*., 117: 771–786

Kakkar, R.K. and V.K. Sawhney, 2002. Polyamine research in plants – a changing perspective. *Physiol. Plant*., 116: 281–292

Kevers, C., T. Gaspar and J. Dommes, 2002. The beneficial role of different auxins and polyamines at successive stages of somatic embryo formation and development of Panax ginseng in vitro. *Plant Cell Tiss. Org. Cult.*, 70: 181–188

Lin, W.C., C.F. Lu, J.W. Wu, M.L. Cheng, Y.M. Lin, N.S. Yang, L. Black, S.K. Green, J.F. Wang and C.P. Cheng, 2004. Transgenic tomato plants expressing the *Arabidopsis NPR1* gene display enhanced resistance to a spectrum of fungal and bacterial diseases. *Transgenic Res*., 13: 567–581

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

Minocha, S.C. and R. Minocha, 1995. Role of polyamines in somatic embryogenesis. *In*: *Somatic Embryogenesis and Synthetic Seed I*, Vol. 30, pp: 53–70. Bajaj, Y.P.S. (eds.). Biotechnology in Agriculture and Forestry. Springer-Verlag publications, Heidelberg, Germany

Mueller, L.A., S.D. Tanksley and J.J. Giovannoni, 2005. The Tomato Sequencing Project, the first cornerstone of the International Solanaceae Project (SOL). *Comparative and Functional Genom.*, 6: 153–158

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

Otoni, W.C., E.A. Picoli, M.G. Costa, F.T. Nogueira and F.M. Zerbini, 2003. *Transgenic Tomato; in Plant Genetic Engineering – a Multivolume Series*, Vol. 5, pp: 41–131. Science Technology Publications, Houston

Park, J., B. Yi and C. Lee, 2001. Effects of plant growth regulators, bud length, donor plant age, low temperature treatment and glucose concentration on callus induction and planter generation in anther culture of cherry tomato 'Mini-Carol'. *J. Kor. Sochort. Sci*., 42: 32–37

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

Qiu, D., G. Diretoo, R. Tavarza and G. Giuliano, 2007. Improved protocol for *Agrobacterium* mediated transformation of tomato and production of transgenic plants containing carotenoid biosynthetic gene *CsZCD*. *Scit. Hortic*., 112: 172–175

Raj, S.K., R. Singh, S.K. Pandey and B.P. Singh, 2005. *Agrobacterium* mediated tomato tansformation and regeneration of transgenic lines expressing tomato leaf curl virus coat protein gene for resistance against TLCV infection. *Curr. Sci.*, 88: 1674–1679

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

Rogozinska, J.H. and L. Skutnik, 1974. Effects of auxins and cytokinins on tomato callus from anthers. *Acta Soc. Bot. Poloniae Warsaw*, 43: 81–92

Sakhanokho, H.F., O.A. Peggy, O.L. May and W.C. Peng, 2005. Putrescine enhances somatic embryogenesis and plant regeneration in upland cotton. *Plant Cell Tiss. Org. Cult*., 81: 91–95

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

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

Youm, J.W., J. Heung, J.H. Jeon, H. Kim, Y.H. Kim, K. Ko, H. Joung and H.S. Kim, 2008. Transgenic tomatoes expressing humanbeta-amyloid for use as a vaccine against Alzheimer's disease. *Biotechnol. Lett*., 30: 1839–1845

Yu, X. and B.M. Reed, 1995. A micropropagation system for hazelnut (Corylus species). *Hortic. Sci.*, 30: 120–123

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