Pak. J. Bot., 48(6): 2489-2498, 2016.

AGROBACTERIUM-MEDIATED TRANSFORMATION AND DIRECT SHOOT REGENERATION IN IRANIAN TOMATO (*SOLANUM LYCOPERSICUM* L.) CULTIVAR FALAT- CH.

NAHEED KAUSER[1,4], SAIFULLAH KHAN[1]*, SEYED ABOLGHASEM MOHAMMADI[2], BEHZAD GHAREYAZIE[3*], EBRAHIM DORANI ULIAIE[2] AND BANAFSHEH DARVISHROHANI[3]

[1]Biotechnology Wing, H.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi- 75270, Pakistan,
[2]Genomics & Molecular Breeding Lab, Department of Plant Breeding & Biotechnology, Faculty of Agriculture, University of Tabriz, Tabriz, 51666 Iran,
[3]Agricultural Biotechnology Research Institute of Iran (ABRII), Karaj, Iran.
[4]Jinnah University for Women, Nazimabad No. 5, Karachi, Pakistan.
[*]Corresponding authors: Email: drsaif65@gmail.com Phone # 92-21-99261693
ghareyazie@yahoo.com Phone#98-2133272234

Abstract

Falat CH is an important commercial tomato cultivar being used in Iran. In this article an optimized protocol with increased transformation and regeneration rate for this tomato variety is reported. Several explants including cotyledon, leaf and hypocotyl were evaluated for direct shoot formation and the effect of various combinations of BAP, Zeatin, IAA and IBA were studied. It is the first report on two cytokinins BAP and Zeatin in various combinations to evaluate the synergetic effect of cytokinins on direct shoot regeneration. The synergetic combination of 1.5mg/l BAP, 0.5 mg/l Zeatin and 0.2 mg/l IAA was considered as the best treatment which resulted in higher plant regeneration rates from all of the explants over previous reported methods. Using the best regeneration treatment obtained, the *HBsAg* gene was transferred into the tomato explants using *Agrobacterium* mediated transformation technique Percent of the putative transgenic plants regenerated was 68%. PCR of putative transformed plants showed that 87.1% of regenerated plants amplified *npt*II and HBsAg gene when specifically designed primers were used; giving a final transformation rate of 34.85%.

Key words: *Agrobacterium tumefaciens*, GV3101, BAP, IAA, IBA, Transformation escapes, Synergetic effect of cytokinins, Zeatin.

Introduction

The advancement in large-scale genomic resource development and recombinant DNA technology for growing number of plants has lead the agricultural scientists towards a new era of developing agronomically improved cultivars using genetic engineering technology. There are thousands of genes that may have to be characterized; one bottleneck to this way is the functional characterization of genes identified by modern technological efforts. One approach that can help in gene function identification is the up /down regulation by transgenic expression. The development of efficient transformation and regeneration protocols for various plant species and cultivars will help in gene characterization.

Solanum lycopersicum L. cultivar Falat CH is a commercially important Iranian cultivar used fresh as well as in the processed form. The increasing growth acreage and commercial value of Falat CH makes it an object for the development of an effective transformation system for the improvement of agronomic traits, quality characteristics and/or molecular farming.

Tomato is one of the well experimented plants especially with respect to the plant regeneration and *Agrobacterium* mediated genetic transformation. The use of a range of explants such as stems, leaves, hypocotyls, cotyledons, apical meristem, stem internodes, anthers and inflorescence has been previously reported for tomato (McCormick *et al.,* 1986; Davis & Miller, 1991; Compton & Veillux, 1991; Jatoi *et al.,* 2001; Raziuddin *et al.,* 2004; Ahmad *et al.,* 2011). A large number of research reports for multiple shoots induction in tomato employing a number of phyto-hormones (PGRs) like Zeatin, Kinetin, BAP and TDZ alone or in combinations with diverse concentrations of auxins are available (Zelcer *et al.,* 1984; Park & Son, 1988; Hamza & Chupeau, 1993; Ye Li & Zhou,1994; Plastira & Perdikaris, 1997; Geetha *et al.,* 1998; Gubis *et al.,* 2003; Shivakumar *et al.,* 2007; Devi *et al.,* 2008; Shadin Ishag *et al.,* 2009; Khan *et al.,* 2013). The success in tomato regeneration response depends on various factors especially the nature of explant, genotype, and Plant growth regulators (PGRs) (Praveen *at el.,* 2011). Therefore, the efficient regeneration protocol is important for *Agrobacterium* mediated transformation in tomato cv. Falat CH.

The responsiveness of various explants for direct shoot initiation and plant regeneration in cultivar Falat CH was evaluated using a large number of media composition reported to be optimal for various genotypes. Besides, we used the combinations of two cytokinins BAP, Zeatin with IAA and IBA to evaluate the synergetic effect of cytokinins on direct regeneration. The optimum medium selected for regeneration of Falat CH was then used to determine the sensitivity assay for Kanamycin selection and direct shoot regeneration capacity of explants infected with *A. tumefascience*. During optimization experiments for genetic transformation, a number of factors were evaluated which influence the transformation efficiency including the impact of light and dark reaction during co-cultivation period. The significance of these factors have

been shown in other plant species, such as *Arabidopsis thaliana* and *Phaseolus acutifolius* (Zambre *et al.,* 2003) but were neglected in tomato transformations.

In present communication we report the development of a reproducible protocol for *Agrobacterium* mediated genetic transformation and direct multiple shoots followed by plantlet establishment in Iranian tomato cv. Falat CH.

Material and Methods

Explant preparation: Seeds of tomato cv. Falat CH were sterilized using 20% commercial bleach (5% NaClO) with 2 drop of Tween-20 for 15 min and rinsed three times with 5min interval in each wash with sterile distilled water. The seeds were cultured on 1/2 strength Murashige & Skoog (MS) (1962) salts medium supplemented with, 20 g /l sucrose and solidified by 2 g /l Phytagel, pH 5.7–5.8. Cotyledons and hypocotyls were excised from 9-19 days germinated plantlets where as leaf explants were cut from 6-8 weeks old *In-vitro* grown tomato plants and pre-cultured for two days.

Direct shoot regeneration and root development: Effect of twenty-nine treatments (combinations of media) were used for direct shoot regeneration and root development of Ch-Falat indicated in Table 1. All media formulations composed of MS salts supplemented with B5 vitamins (Gamborg *et al.,* 1968), 30 g/l sucrose and various combinations of plant growth regulators (BAP, Zeatin, IAA, IBA and NAA). 2.5 g/l of Phytagel was used as solidifying agent.

Leaf and Cotyledonary explants were placed upside down on medium where as hypocotyls were placed horizontally. The plates were incubated at 25 ± 2ºC under white fluorescent lights with 16/8 hrs photoperiod .After 3 weeks, the induction of calli and shoot primordia was recorded. After 4 weeks shoots or shoot primordia were recounted, recorded (Table 1) and transferred to fresh medium.

Generally non-transformed tomato shoots develop roots on MS medium with 30 g/l sucrose. In case of transformed shoots it was observed that majority of plantlets failed to develop root on MS medium. To optimize the rooting of transformed shoots various combinations of three auxins IAA, IBA and NAA were used on two strengths of MS basal medium i.e. full and half strength supplemented with 20 g/l sucrose and solidified with 0.25 % phytagel (Table 2).

Table 1. The effect of different combinations and concentrations of plant growth regulators on shoot development from various explants of Falat CH.

Treatment	BAP (mg l^{-1})	ZEATIN (mg l^{-1})	IAA (mg l^{-1})	IBA (mg l^{-1})	Shoots developed per explant		
					Hypocotyl	Cotyledon	Leaf
T1	-	-	-	-	0.0 ± 0.0 M	0.0 ± 0.0 T	0.0 ±0.0 M
T2	1	-	0.1	-	6.3 ± 0.11 HI	8.3 ± 0.17 P	7.5 ±0.16 IJ
T3	2	-	0.1	-	9.0 ± 0.11 D	11.0 ± 0.14 K	10.0 ±0.2 FG
T4	1	-	0.5	-	5.0 ± 0.11 KL	7.0 ± 0.18 RS	6.0 ±0.12 L
T5	2	-	0.5	-	7.3 ± 0.11 FG	9.3 ± 0.15 MN	9.0 ±0.12 H
T6	1	-	-	0.1	5.2 ± 0.10 K	7.2 ± 0.15 R	7.0 ±0.18 JK
T7	2	-	-	0.1	8.1 ± 0.11 E	10.1 ± 0.14 L	9.0 ±0.16 H
T8	1	-	-	0.5	4.5 ± 0.11 L	6.5 ± 0.19 S	6.5 ±0.18 KL
T9	2		-	0.5	6.5 ± 0.10 HI	8.5 ± 0.19 OP	8.0 ±0.12 I
T10	-	1	0.1	-	7.9 ± 0.11 EF	9.9 ± 0.15 LM	9.5 ±0.12 GH
T11	-	2	0.1	-	12.1 ± 0.1 A	13.0 ± 0.14 I	14.0 ±0.18 A
T12	-	1	0.5	-	5.9 ± 0.11 IJ	7.9 ± 0.15 PQ	7.0 ±0.15 JK
T13	-	2	0.5	-	8.0 ± 0.10 EF	10.0 ± 0.25 L	9.5 ±0.15 GH
T14	-	1	-	0.1	7.0 ± 0.10 GH	9.0 ± 0.16 NO	8.0 ±0.11 I
T15	-	2	-	0.1	10.0 ± 0.11 C	12.0 ± 0.25 J	11.0 ±0.15 DE
T16	-	1	-	0.5	5.3 ± 0.11 JK	7.3 ± 0.16 QR	6.0 ±0.18 L
T17	-	2	-	0.5	9.0 ± 0.10 D	11.0 ± 0.25 K	10.5 ±0.18 EF
T18	1	0.5	0.2	-	9.0 ± 0.11 D	19.0 ± 0.17 D	11.0 ±0.25 DE
T19	1.5	0.5	0.2	-	11.0 ± 0.12 C	25.0 ± 0.33 A	14.5 ±0.27 A
T20	2	0.5	0.2	-	10.0 ± 0.11 C	21.0 ± 0.4 C	13.0 ±0.2 B
T21	1	1	0.2	-	10.2 ± 0.10 C	17.0 ± 0.42 E	12.0 ±0.22 C
T22	1.5	1	0.2	-	9.0 ± 0.11 D	15.0 ± 0.53 G	12.0 ±0.3 C
T23	2	1	0.2	-	8.0 ± 0.10 EF	13.0 ± 0.33 I	10.0 ±0.25 FG
T24	1	0.5	-	0.2	7.8 ± 0.11 EF	17.0 ± 0.4 E	9.0 ±0.12 H
T25	1.5	0.5	-	0.2	10.2 ± 0.11 C	22.0 ± 0.53 B	14.0 ±0.18 A
T26	2	0.5	-	0.2	9.6 ± 0.11 CD	19.0 ± 0.2 D	12.0 ±0.2 C
T27	1	1	-	0.2	9.0 ± 0.11 D	16.0 ± 0.4 F	11.5 ±0.12 CD
T28	1.5	1	-	0.2	8.0 ± 0.11 EF	14.0 ± 0.33 H	11.0 ±0.18 DE
T29	2	1	-	0.2	7.0 ± 0.12 GH	11.0 ± 0.33 K	9.0 ±0.19 H

All means ± standard error are compared by DMRT at p=0.1. Values followed by the same letter are not significantly different from each other

Table 2. The effect of different combinations and concentrations of Auxins and strength of MS salts on root development.

Treatment	IAA	IBA	NAA	Full MS medium		½ MS medium	
	(mg l^{-1})			No. of roots	Root length	No. root	Root length
R1	-	-	-	1.0 ± 0.39 L	4.0 ± 0.62 FG	2.0 ± 0.37 H	5.0 ± 0.29 EFG
R2	0.1	-	-	3.0 ± 0.6 K	5.0 ± 0.83 EF	6.0 ± 0.18 G	7.0 ± 0.25 BC
R3	0.5	-	-	7.0 ± 1.49 HIJ	6.5 ± 1.14 CD	10.0 ± 0.31 F	8.0 ± 0.70 B
R4	1	-	-	13.0 ± 2.24 C	6.5 ± 1.14 CD	15.0 ± 0.31 D	7.0 ± 0.35 BC
R5	1.5	-	-	16.0 ± 2.85 B	7.0 ± 1.18 C	17.0± 0.25BC	9.0 ± 0.37 A
R6	2	-	-	11.0 ± 1.67 DE	6.0 ± 1.01 CDE	13.0 ± 0.31 E	5.5 ± 0.4 DEF
R7	-	0.1	-	6.0 ± 0.86 J	5.0 ± 0.83 EF	6.0 ± 0.31 G	5.0 ± 0.31 EFG
R8	-	0.5	-	10.0 ± 1.65 EF	8.0 ± 1.37 B	10.0 ± 0.33 F	7.9 ± 0.22 B
R9	-	1	-	16.0 ± 2.79 B	9.0 ± 1.62 A	18.0 ± 0.29 B	10.0 ± 0.24 A
R10	-	1.5	-	19.0 ± 3.44 A	9.2 ± 1.59 A	20.0 ± 0.4 A	10.0 ± 0.31 A
R11	-	2	-	15.0 ± 2.47 B	7.0 ± 1.19 C	15.0 ± 0.31 D	8.0 ± 0.18 B
R12	-	-	0.1	4.0 ± 0.61 K	5.0 ± 0.74 EF	5.0 ± 0.31 G	5.0 ± 0.25 EFG
R13	-	-	0.5	7.0 ± 1.14 HIJ	5.5 ± 0.98 DE	9.0 ± 0.31 F	6.0 ± 0.25 CDE
R14	-	-	1	12.0 ± 2.08 CD	3.9 ± 0.70 G	16.0 ± 0.3 CD	4.0 ± 0.12 GH
R15	-	-	1.5	13.0 ± 2.47 C	3.0 ± 0.50 G	16.0 ± 0.31CD	3.3 ± 0.23 H
R16	-	-	2	9.0 ± 1.70 FG	1.2 ± 0.31 H	12.0 ± 0.31 E	2.0 ± 0.2 I
R17	0.1	0.2	-	8.0± 1.35 GHI	3.5 ± 0.70 G	12.0 ± 0.70 E	4.7 ± 0.16 FG
R18	0.1	-	0.2	8.5± 1.53 FGH	5.4 ± 0.98 E	12.0 ± 0.70 E	6.5 ± 0.25 CD
R19	0.1	0.1	0.1	6.5 ± 1.17 IJ	6.0 ± 1.11 CDE	9.0 ± 0.31 F	6.5 ± 0.2 CD

All means ± standard error are compared by DMRT at p=0.1. Values followed by the same letter are not significantly different from each other

Kanamycin sensitivity assay: To determine sensitivity of various explants of Falat CH to Kanamycin; explants were tested in T19 medium (Table 1) which showed the highest regeneration potential irrespective of explants type with various Kanamycin concentrations: 0, 25, 50,75,100 and 125mg/l and sub- cultured every 15 days.

Preparation of *Agrobacterium tumefaciens*: *Agrobacterium tumefaciens* GV31011 harboring plant binary vector pHB117(courtesy to Hugh S. Mason), with CaMV 35S double promoter for constitutive expression of Hepatitis B surface Antigen (HBsAg) and neomycin phosphortransferase II (*npt*II) genes, was used in transformation experiments. A single colony of freshly grown *A. tumefaciens* was inoculated in 5 ml of YEP broth supplemented with 50 mg/l kanamycin and 25 mg/l rifampicin and grown at 200 rpm at 28ºC for 24 h. The 1 ml of overnight grown culture was added to 25 ml of YEP broth with specified antibiotics and cultured for 4–5 hrs until an O.D. reached to 0.8 - 1.0. The 10 ml of bacterial culture was centrifuged for 10 min at 4ºC with the speed of 4,000 rpm. Bacterial pallet was suspended in liquid T1 medium with various concentrations (0.0, 50, 100, 150, 200, 250 and 300) µM of acetosyringone.

Co-cultivation and light test: The 2 days old pre-cultured explants were inoculated in the *A. tumefaciens* suspension for 10, 20, 30, 40, 50 and 60 minutes with slight shaking on gyratory shaker. They were removed from the suspension, then blot dried and transferred to T1 medium supplemented with various levels (0.0, 50, 100, 150, 200, 250 and 300) µM of acetosyringone. To determine the effect of light, some cultures co- cultivated in dark and some in light for 1, 2 and 3 days on previously mentioned temperature conditions.

Concentration of acetosyringone: The concentrations of acetosyringone were selected on previous reports about transformation of mono cotyledonous plants using *Agrobacterium tumefaciens* (Dong & Qu, 2005). Leaf segments were used as target tissues for transformation assays.

Transformant Selection and plant regeneration: To eliminate *A. tumefaciens* overgrowth and to maintain a strict selection, the co-cultivated explants were transferred to T19 medium containing 125mg/l Kanamycin and 500 mg/l of cefotaxime and were sub-cultured every 14 days.

Four to six weeks later, regenerated shoots/ primordia were excised and transferred onto fresh medium supplemented with 50 mg/ l kanamycin and 250 mg/l cefotaxime. Few shoots produced roots in 2-4 weeks. Large no. of shoots that failed to produce roots on same medium were transferred to rooting medium R10 (Table 2) supplemented with 50 mg/l Kanamycin. After generation of roots, plants were transferred to soil and acclimatized in growth room (25 ± 2ºC, 16/8 photoperiod). Plants were then transferred to a greenhouse to grow till maturity.

Molecular analysis of putative transgenic plants using polymerase chain reaction (PCR): Genomic DNA from controlled and transformed Falat CH leaves was extracted followed by the method of Doyle and Doyle (1990). Transformation analysis was carried out by PCR employing specific primers designed for the selectable marker gene *nptII* and HBsAg.

A 700 bp fragment of HBsAg gene was amplified by PCR using a pair of gene specific primers, Forward (5´ CGACCATGGAGAACACAACA 3´) and Reverse (5´ AGACTTAGATGTACACCCAAAGACAA 3´) under thermo-cycler program, which consisted of 5 min-at 94ºC

for initial denaturing, followed by 35 cycles of 30 sec at 94°C, 30 sec at 62°C, 45 sec at 72°C, and finally for 7 min-at 72°C for extension.

The following primers pair was used to detect the presence of *nptII* gene: Forward (5´ GAGGCTATTCGGCTATGACTG 3´) and reverse (5´ TAGAAGGCGATGCGCTGCGA 3´). PCR profile was consisted of an initial denaturation of 4 minutes at 95°C, followed by 35 cycles of 30 sec at 95°C, 1 minute at 56°C and 1 minute at 72°C, with a final extension of 5 minutes at 72°C. A fragment of approx. 730bp was expected to be amplified. In both cases, the reaction was carried out with 100ng of the template DNA. The amplified product were separated by electrophoresis on 1.2% agarose gel and visualized under UVITECH gel documentation system.

Statistical analysis: Direct shoot development experiments were conducted with two factors [Explant type and treatments (medium composition)] in a randomized complete block design (RCBD) (15 explants/plate; 5 replications/treatment). The data was analyzed by two-way ANOVA with replications. Root development experiments were tested in a randomized complete block design (10explants/plate; 5 replications /treatment) and were analyzed by two ways ANOVA. Factor one was treatments (auxins) and factor two was strength of media (1/2 and full MS). Each dish was considered as a replicate in all the experiments designed. Differences between the means were obtained using Duncan's Multiple Range Test with 0.1 confidence level. All analysis was done using a DOS based statistical software MSTATC.

Results and Discussion

Plant regeneration and root development: Various PGR treatments (in combination or alone BAP, Zeatin, IAA and IBA) were evaluated for direct regeneration, the swelling of explants and some callus initiation was observed at the cut edges within 5–10 days of culture. After 2 weeks of culture, granular green callus and leaf primordia were produced from cotyledonary and leaf explants on all treatments, except for T1 which included no PGR and served as control. The callus response exhibited by hypocotyl segments was greater for the IBA combinations than for the IAA combinations (Data not shown here).

Two to three weeks after initiation, the well-developed regenerated shoots were noted for the evaluation of the best treatment. The most of the multiple shoots were developed directly from the cut ends of explant (Fig. 1b). Shoots were developed directly from all treatments T2-T29 with or without some callus. Significant differences appeared in explants producing shoot primordia on various treatments, and an interaction between explant type and treatments was also observed (Table 3 (ANOVA)). The data showed that the T19 treatment (BAP 1.5 mg/l, Zeatin 0.5 mg/l and IAA 0.2 mg/l) was the best for the generation of maximum number of direct shoots from single explants on average irrespective of type of explants. The T8 Treatment (BAP 1.0 mg/l and IBA 0.5 mg/l) proved the least effective treatment for direct shoot development as it produced 5 shoots per explant (Fig. 3) only. The control T1 treatment showed no effect on explants and developed no shoot in any explants.

The type of the explants showed the significant difference on shoot development. Cotyledonary explants proved to be the best for regeneration efficiency as it developed up to 25 shoots per explant, the leaf explants showed the average maximum number of 14.5 shoots per explant where as the hypocotyls developed only 12 shoots per explant (Table 1). This finding is in concordance of previous investigations which demonstrated that cotyledons of tomato were more efficient than other sources of explants, including leaves, hypocotyls and stems for shoot organogenesis (Hamza and Chupeau, 1993; Van Roekel *et al.*, 1993; Ling *et al.*, 1998).

Treatment T19 (combination of 1.5 mg/l BAP, 0.5mg/l Zeatin and 0.2 mg/l IAA) and the T25 treatment (1.5 mg/l BAP, 0.5mg/l Zeatin and 0.2 mg/l IBA) produced highest number of shoots from cotyledonary explants (25.0±0.33 and 22.0 ± 0.53 shoots from single explant respectively) (Table 1). Treatment T11 (2.0mg/l Zeatin and 0.1 mg/l IAA) showed the highest number of shoots produced from hypocotyls (12.1±0.1 shoots per explant). These findings are in agreement with the report of Gubis *et al.* (2003) who reported that the medium fortified with Zeatin and IAA was the most effective in induction of adventitious shoots in tomato cultivar Premium. When the hypocotyls were tested with T19 treatment, the average shoots produced were 11.0±0.12. The leaf explants produced the maximum number of shoots 14.5±0.27 in T19 like cotyledons.

The highest percentage of response and maximum frequency for number of multiple shoots formation in all explants were found at the treatments containing BAP and Zeatin in combination with IAA or IBA. This result showed that the synergetic effect of cytokinins BAP and Zeatin is more effective for direct organogenesis in tomato as compared to the single one. Though, the treatments which were comprised on single cytokinins in combination with low concentration of auxins are effective to produce shoots yet so synergetic effect of two cytokinins with low concentration of single auxin show higher significantly (Table 1, Fig. 3). Nogueira *et al.* (2001) also reported that lower concentrations of IAA with cytokinins enhanced the shoot development in tomato cultivar Santa Clara. In contrast to previously report by Afroz *et al.* (2010) showed that organogenesis ability of explant decreased drastically after transformation. The combination of PGRs in T19 treatment gave good number of transformed shoots irrespective of explant type and genotype. Sheeja *et al.* (2004) and Rizwan Rasheed *et al*. (2010) also reported the use of two cytokinins BAP and Kinetin in combination for the direct shoot development in tomato with maximum 4.78 shoots per explant.

For *In vitro* rooting, the shoots were cultured on 1/2 strength MS, full strength MS medium supplemented with various concentrations of IAA, IBA and NAA separately (Table 2). Root formation was initiated within 10-12 days after inoculation in all treatments and in control (medium without any PGR). Mensuali Sodi *et al.* (1995) reported the *In vitro* rooting of tomato does not require any PGR_S, which supports our findings. The highest average number of roots 20.0 ±0.4 was observed on ½ MS supplemented with 1.5 mg/l IBA with profuse rhizogenesis (Fig. 1d). This finding is in line with Sheeja *et al.* (2004) report on the best effect of half strength of MS on root development.

The present study showed the effect of different auxins on rooting on 1/2MS medium compared to an auxin-free medium which supported the promotive effect of auxins on root initials (De Klerk *et al.*, 1999) in tomato.

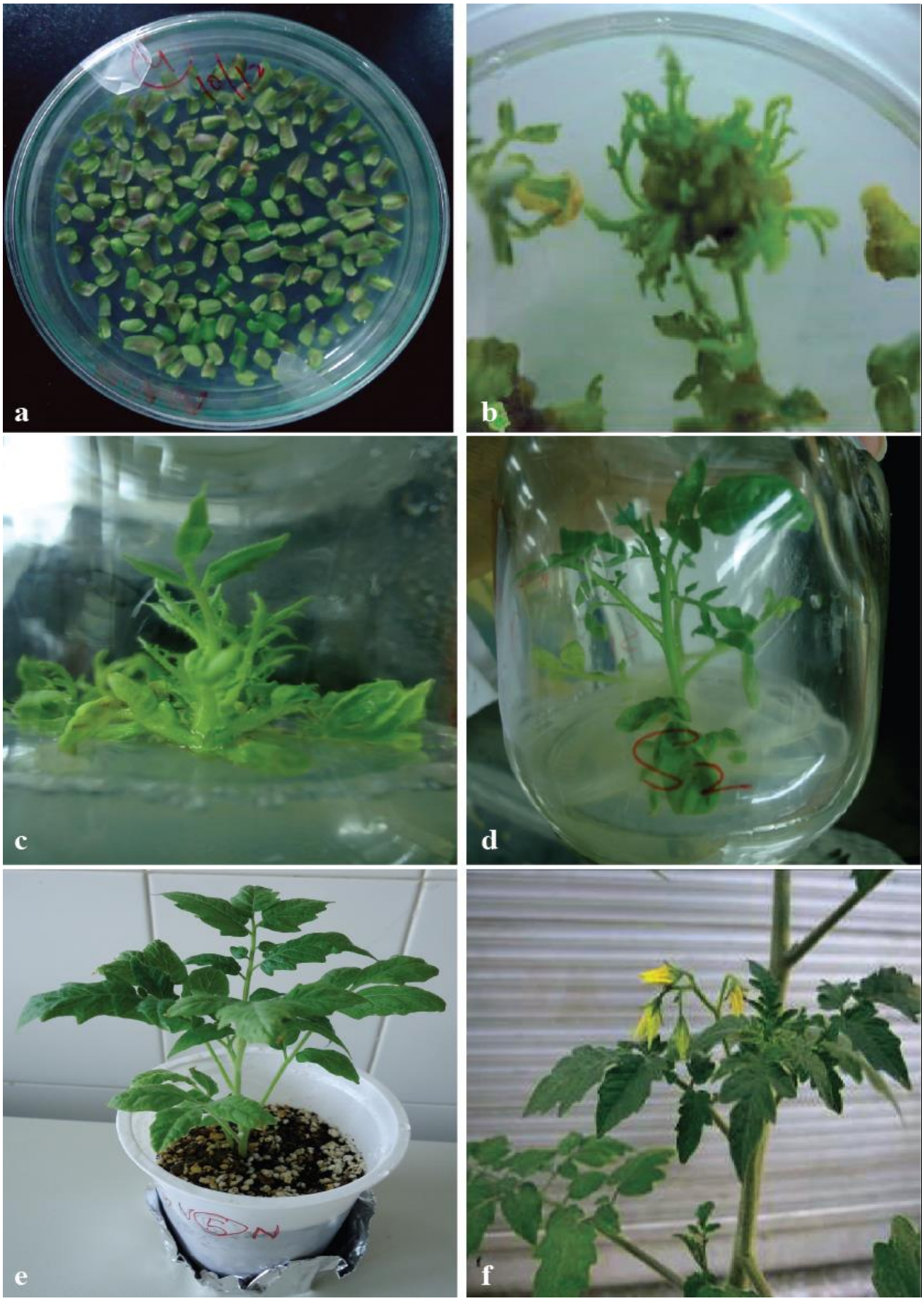

Fig. 1. Various stages of *Agrobacteriuum* mediated transformation of Falat CH. (a) Co-cultivation of cotyledons (b) Direct organogenesis (c) Shoot development (d) Rooting (e) Acclimatization (f) Flowering on transformed plant.

Table 3a. ANOVA for shoot development.

K value	Source of variation	Degree of freedom	Sum of squares	Mean square	F-value	Prob
1	Replication	4	17.800	4.450	28.6800	0.0000
2	Factor A	36	6430.130	178.615	1151.1426	0.0000
4	Factor B	2	1530.542	765.271	4932.0457	0.0000
6	AB	72	1362.044	18.917	121.9187	0.0000
-7	Error	440	68.272	0.155		
	Total	554	9408.787			

Table 3b. ANAOVA for no. of roots.

K value	Source of variation	Degree of freedom	Sum of squares	Mean square	F-value	Prob
1	Replication	4	1.324	0.331	0.4305	0.0000
2	Factor A	18	4173.903	231.883	301.6336	0.0000
4	Factor B	1	136.426	136.426	177.4631	0.0013
6	AB	18	34.724	1.929	2.5094	
-7	Error	148	113.776	0.769		
	Total	**189**	4460.153			

Table 3c. ANAOVA for root length.

K value	Source of variation	Degree of freedom	Sum of squares	Mean square	F-value	Prob
1	Replication	4	3.554	0.889	2.5379	0.0424
2	Factor A	18	698.595	38.811	110.8501	0.0000
4	Factor B	1	25.642	25.642	73.2386	0.0000
6	AB	18	21.794	1.211	3.4581	0.0000
-7	Error	148	51.818	0.350		
	Total	**189**				

Kanamycin assay and selection for transformed plants: Our results in line with the reports from previous studies indicates that cotyledon is a good choice as explant for good regeneration in tomato (Fillati *et al.,* 1987; Hamza *et al.,* 1993; Frary *et al.,* 1996; Ellul *et al.,* 2003; Sharma *et al.,* 2009), therefore, we used cotyledon as explant for optimization of transformation of Falat CH.

To determine the kanamycin concentration for transformed shoots selection, explants were cultured on medium supplemented with a range of antibiotic concentrations (Fig. 4). *In vitro* shoot regeneration ability of Falat CH was approximately 98% in T19 medium devoid of Kanamycin. Initially, some explants formed shoot primordia and calli on 50–75 mg/l kanamycin, but failed to develop normal shoots and died after three weeks. It was observed that 100 and 125 mg/l kanamycin inhibited the regeneration in Falat CH completely. Therefore, 125 mg/l Kanamycin was chosen as the selection threshold for transformed shoots.

To avoid the problem of transformation escapes reported by others on various plant species (Dong *et al.,* 1991; Akasaka *et al.,* 2004; Wu *et al.,* 2009). We decreased the size of explant (cotyledon cut into 3-4 pieces) and replaced fresh medium supplemented with appropriate selection after every two weeks. The small size of explants reduced the curling of cotyledonary and leaf explants, where as replacing selection medium frequently exerted continuous high selection pressure which resulted in no escape.

Co-cultivation: In the beginning, various *A. tumefaciens* strains LBA4404, AGL1, GV3101 and EHA105 were tested to determine their transformation ability with various explants of five tomato genotypes. LBA 4404 and GV3101 showed fairly good transformation efficiencies (Data not shown). GV3101 was used in subsequent studies for Falat CH as it was proved to be more efficient in producing stable transformants compared to LBA4404 (Data not shown).

Co-cultivation is one of the critical steps in transformation experiments. It influences the transformation efficiency (Fang *et al.,* 1990; Fullner and Nester 1996; Shilpa *et al.,* 2010; Yasmin *et al.,* 2010; Seo *et al.,* 2011; Sharma *et al.,* 2011), Our findings suggested that co-cultivation at 24^{o}C for 76 h with *A. tumefaciens* strain GV301 results in the overgrowing of the bacteria and the necrosis of explants which led to the low transformation efficiency. Co- cultivation for 48 h was proved optimal at 24±2^{o}C for Falat CH cotyledons and 13 explants out of 15 were transformed (Fig. 5). This result is contradictory to Sharma *et al.* (2009) who reported that 72 h of co-cultivation is optimum for higher transformation but in agreement with other reports to achieve maximum transformation efficiency in various cultivars of tomato

with various *Agrobacterium* strains (LBA4404, C58C1, GV311SE or A208) by 48 h co-cultivation interval (McCormick *et al.,* 1986; Hamza *et al.,* 1993; Lipp-Joao *et al.,* 1993; Frary *et al.,* 1996; Oktem *et al.,* 1999; Vidya *et al.,* 2000; Pozueta-Romero *et al.,* 2001; Jia *et al.,* 2002; Ellul *et al.,* 2003).

Light/dark effect: Zambre *et al.* (2003) reported a constructive effect of light on gene transfer from *A. tumefaciens* to root explants of *Arabidopsis thaliana* and callus of *Phaseolus acutifolius*. However, we did not observe any significant differences in transformation with dark and light treatments during co-cultivation (Table 5). It is possible that the light/dark effect of *Agrobacterium*-mediated transformation may be genotype or explant type (callus/root) or co- cultivation temperature dependent.

Acetosyringone (As) concentration: Acetosyringone (As) is a phenolic compound and is used to induce the vir genes of the bacteria for the transformation of monocots and recalcitrant dicot plant species (Satchel *et al.,* 1985). It has been reported that the addition of As to co-cultivation medium improves the *Agrobacterium*-mediated transformation in several plant species (Svabova & Griga, 2008; Wang *et al.,* 2009). Various reports have suggested that some tomato cultivars that are recalcitrant to transformation improved the efficiency many folds with the application of As (Costa *et al.,* 2006; Afroz *et al.,* 2010; Sharma *et al.,* 2011).

In present study optimum transformation was obtained with 200µM of As whereas higher concentrations lead to the death of explant by overgrowth of *Agrobacterium*. Lower concentrations 50 - 100 µM of As did not have significant effect to the transformation of Falat CH (Fig. 6). This result supports the finding of Priya & Shivendra (2009) and Tian Li *et al.* (2011) who reported 100 mg/l and 200 mg/l As significantly improved tomato transformation efficiency.

Molecular analysis of transformed plants by PCR: All plants rooted on Kanamycin selection medium were screened for the presence of *npt*II and HBsAg genes. Genomic DNA from Kanamycin resistant plants amplified a 700bp fragment of the HBsAg gene (Fig. 2a) and 730 bp fragment of *npt*II gene (Fig. 2b). Regenerated non-transformed control plants failed to amplify any fragment. Most non-transformed shoots were killed by kanamycin selection. Based on PCR analysis, we found only 9 Falat CH regenerated plant to be non-transgenic escapes out of 70.

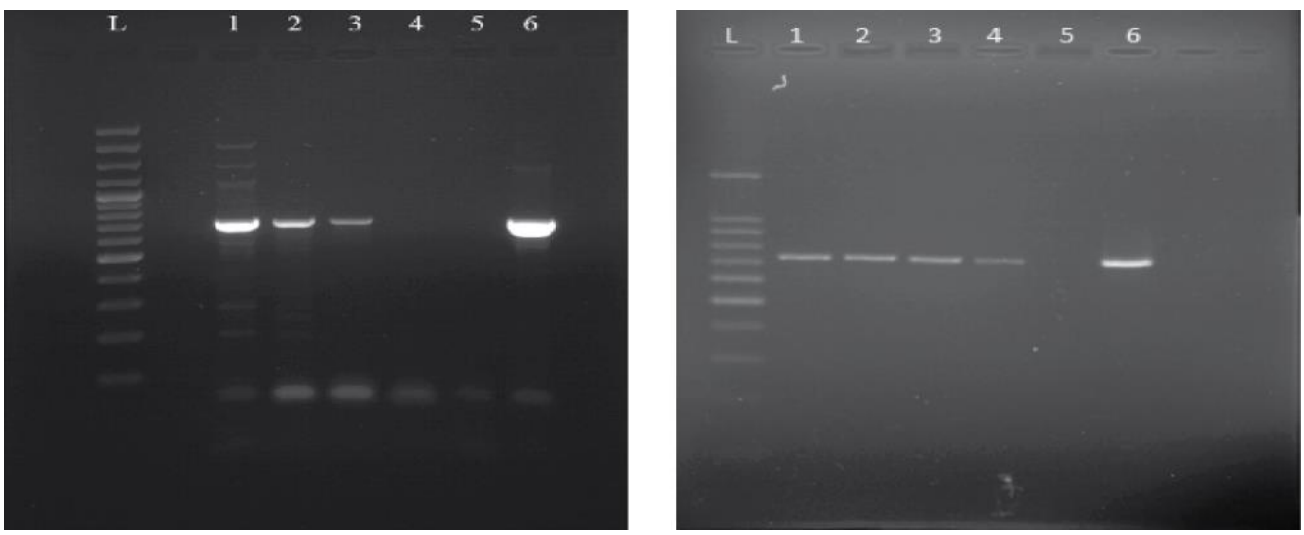

Fig. 2. (a) PCR amplification of 700 bp HBsAg gene and (b) 730 bp *nptII* gene; L is 100bp *Fermentas gene ruler* ladder, lane 1-4 transformed Plants, lane 5 untransformed control and lane 6 plasmid DNA.

Table 4. Regeneration percentage and transformation efficiency of Falat CH.

No. of explants	Shoot regeneration	Shoot regeneration %	Kanamycin resistant shoots	PCR positive shoots	Transformation efficiency %
175	119	68%	70	61	51.2%

Transformation Efficiency is calculated by the ratio between regenerated shoots and PCR positive shoots

Table 5. Impact of light /dark condition on transformation efficiency.

Light condition	No. of explants	No. of Kanamycin resistant explants	PCR positive	Transformation % age
Dark	35	25	22	88%
Light	35	27	24	88.88%

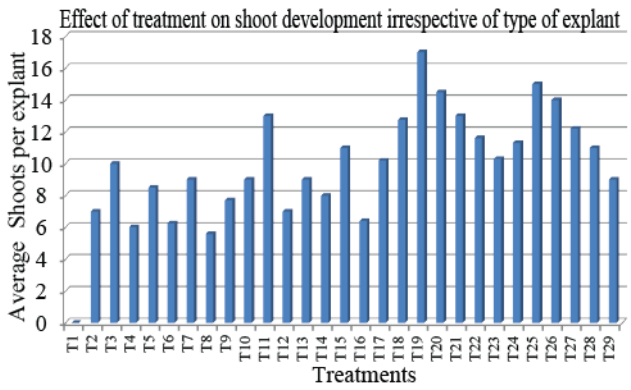

Fig. 3. Overall effect of PGR treatment on Falat CH irrespective of explant type.

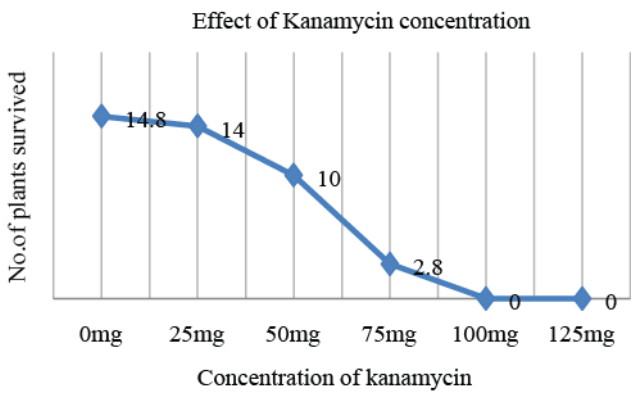

Fig. 4. Effect of Kanamycin conc. on transformation.

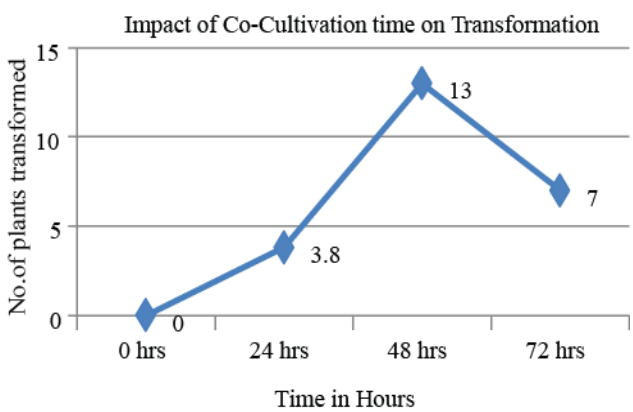

Fig. 5. Impact of co-cultivation time on transformation.

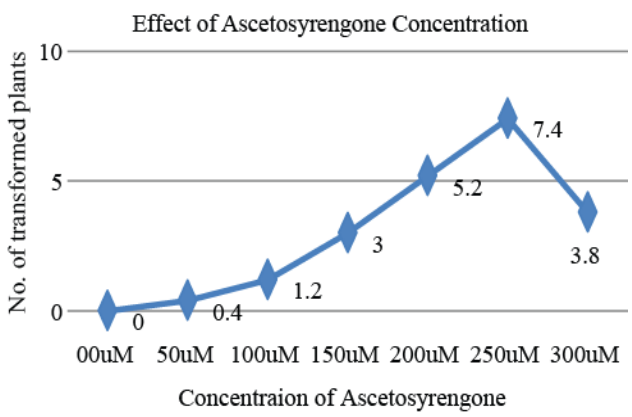

Fig. 6. Effect of Ascetosyringone concentration on transformation.

Conclusion

In present study total 175 cotyledon explants were used for the onset of *Agrobacterium* infection, during first three weeks 119 explants developed the shoots and some of them died with continuous selection pressure during first three weeks, this showed that regeneration ability of Falat CH is 68% after transformation (Table 4). Seventy (70) shoots developed roots on Kanamycin selection medium. These plants were tested for the presence of *npt*II. 61 out of 70 plants showed the presence of Kanamycin resistance gene. This confirmed that escape event is very low in this protocol as 87.1% of developed shoots were carrying the selection gene. Overall transformation percentage was 34.85% where as transformation efficiency with respect to regenerated shoots was 51.2% (Table 4).

Acknowledgement

We acknowledge the support of International Centre for Chemical and Biological Sciences, University of Karachi, Pakistan and Genomics & Molecular Breeding Lab, Department of Plant Breeding & Biotechnology, Faculty of Agriculture, University of Tabriz, Iran for execution of this research work .We are thankful to Dr. Hugh S. Mason, School of life sciences, Arizona State University for providing us the plasmid pHB117; Dr. Reza Dadpour for environment controlled growth chambers; Mr. Habib Kazimia for gardening guidance and Mr. Amir Kahnamoei for providing technical and lab support.

References

Afroz, A., Z. Chaudhry, U. Rashid, G.M. Ali, F. Nazir, J. Iqbal and M.R. Khan. 2010. Enhanced resistance against bacterial wilt in transgenic tomato (*Lycopersicon esculentum*) lines expressing the Xa21 gene. *Plant Cell Tiss. Org. Cult*., 104: 227-237.

Ahmad, N., B. Guo, H. Fazal, B.H. Abbasi, C.Z. Liu, T. Mahmood and Z. K. Shinwari. 2011. Feasible plant regeneration in black pepper from petiole explants. *J.M.P.R*., 5(18): 4590-4595.

Akasaka-Kennedy, Y., K. Tomita and H. Ezura. 2004. Efficient plant regeneration and *Agrobacterium*-mediated transformation via somatic embryogenesis in melon (*Cucumis melo* L.). *Plant Sci*., 166: 763-769.

Branca, C., A. Torelli, P. Fermi, M. Altamura and M. Bassi. 1994. Early Phases *In vitro* culture of tomato cotyledons: starch accumulation and protein pattern in relation to the hormonal treatment. *Protoplasma*, 182: 1-2.

Compton, E., D.J. Gray and V.P. Gaba. 1991. Genetic transformation of watermelon. In: Curtis IS (ed.) *Transgenic crops of the world essential protocols*. Kluwer Academic Publishers, Dordrecht, pp. 425-433.

Costa, M.S., C. Miguel and M.M. Oliveira. 2006. An improved selection strategy and the use of acetosyringone in shoot induction medium increase almond transformation efficiency by 100-fold. *Plant Cell Tiss. Org. Cult.*, 85: 205-209.

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.

De Klerk, G.J., W.M. van Der Krieken and J.C. De Jong. 1999. The formation of adventitious roots: new concepts, new possibilities. *In vitro Cell Dev. Biol. Plant*., 35: 189-199.

Devi, M., M.S. Dhaliwal, A. Kaur and S.S. Gosal. 2008. Effect of growth regulators on *In vitro* morphogenetic response of tomato. *Ind. J. Biotechnol.,* 7: 526-530.

Dong, J.Z., M.Z. Yang, S.R. Jia and N.H. Chua. 1991. Transformation of melon (*Cucumis melo* L.) and expression from the cauliflower mosaic virus-35 s promoter in transgenic melon plants. *Biotechnology*, 9: 858-863.

Dong, S. and R. Qu. 2005. High efficiency transformation of tall fescue with *Agrobacterium tumefaciens*. *Plant Sci.,* 186: 1453-1458.

Doyle, J.J. and Doyle J.L. 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.

Fang, G. and R. Grumet. 1990. *Agrobacterium tumefaciens* mediated transformation and regeneration of muskmelon plants. *Plant Cell Rep*., 9: 160-164.

Fillati, J.J., J. Kiser, R. Rose and L. Comai. 1987. Efficient transfer of a glyphosate tolerance gene into tomato using a binary *Agrobacterium tumefaciens* vector; *Biotechnology*, 5: 26-731.

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.

Fullner, K.J. and E.W. Nester. 1996. Temperature affects the T-DNA transfer machinery of *Agrobacterium tumefaciens*. *J. Bacteriol.,* 178: 1498-1504.

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.

Geetha, N., P. Venkatachalam, P.S. Reddy and G. Rajaseger. 1998. *In vitro* plant regeneration from leaf callus cultures of tomato (*Lycopersicon esculentum* Mill). *Adv. Plant Sci.,* 11: 253-257.

Gubis, J., Z. Lajchova, J. Farago and Z. Jurekova. 2003. Effect of genotype and explant type on shoot regeneration in tomato (*Lycopersicon esculentum* Mill.) *In vitro*. *Czech J. Genet. Plant Breed.,* 39: 9-14.

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

Jatoi, S, A, G.M. Sajid, H. Sappal, M.S. Quraishi and R. Anwar. .2001. Differential *In vitro* response of tomato hybrids against a multitude of hormonal regimes. *J. Biol. Sci*., 1: 1141-1144.

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

Khan, M.A., B.H. Abbasi and Z.K. Shinwari. 2014. Thidiazuron enhanced regeneration and silymarin content in *Silybum marianum* L. *Pak. J. Bot.,* 46(1): 185-190.

Ling, H.Q., D. Kriseleit and M.W Ganal. 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 T.A. Brown. 1993. Enhanced transformation of tomato co-cultivated with *Agrobacterium tumefaciens* C58C1Rifr:pCSFR1161 in the presence of acetosyringone. *Plant Cell Rep*., 12: 422-425.

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

Mensuali-Sodi, A., M. Panizza and F. Tognoni. 1995. Endogenous ethylene requirement for adventitious root induction and growth in tomato cotyledons and lavaandin microcuttings *In vitro*. *Plant Growth Reg.,* 17: 205-212.

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

Nogueira, F.T.S., M.G. Costa, M.L. Figueira, W.C. Otoni and F.L. Finger. 2001. *In vitro* regeneration of Santa Clara" tomato plantlets and its natural mutant Firme". *Scien. Agrotec. Lavras.,* 25: 36-71.

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

Park, Y.G. and S.H. Son. 1988. *In vitro* organogenesis and somaticembryogenesis from punctured leaf of *Populus nigra P. maximowiezii. Plant Cell Tiss. Org. Cult.,* 1595-1105.

Plastira, V.A. and A.K. Perdikaris. 1997. Effect of genotype and explant type in regeneration frequency of tomato *In vitro*. *Acta Horti*., 231-234.

Pozueta-Romero, J., G. Houlne, L. Canas, R. Schantz and J .Chamarro. 2001. Enhanced regeneration of tomato and pepper seedling explants for *Agrobacterium*-mediated transformation. *Plant Cell Tiss. Org. Cult.* 67: 173-180.

Praveen, M. and R.S. Nanna. 2011. Effect of genotype, explant source and medium on *In vitro* regeneration of tomato. *Int. J. Genet. and Mol. Biol.,* 3(3): 45-50.

Priya, P. and V.S. Shivendra. 2009. Genetic transformation and regeneration of *Sesbania drummondii* using cotyledonary nodes. *Plant Cell Rep*., 28: 31-40.

Raziuddin, S.S.., H.J. Chaudhary, T. Mohammad and S. Ali. 2004. Hormonal effect on callus induction in tomato. *Sarhad. J. Agri*. 20: 223-225.

Rizwan, R., S.S. Bal, I.O. Hamidullah, A. Kaur and J.A. Bhat. 2010. Effect of growth Regulators on direct shoot regeneration in Tomato. *Int. J. of Biotech. and Biochem.,* 6(1): 81-86.

Sambrook, J., E.F. Fritsch and T. Maniatis. 1989. *Molecular cloning*. A laboratory manual 2nd edition (New York: Cold Spring Harbor Laboratory Press).

Satchel, S.E., E. Messens, M.V. Montagu and P. Zambryski. 1985. Identification of the signal molecules produced by wounded plant cells that activate T-DNA transfer in *Agrobacterium rhizogenes*. *Nature,* 318: 624-629.

Seo, M.S., S. Takahashi, K. Kadowaki, M. Kawamukai, M. Takahara and T. Takamizo. 2011. Expression of CoQ10-producing dds DNA transgene by efficient *Agrobacterium*-mediated transformation in *Panicum meyerianum*. *Plant Cell Tiss. Org. Cult.,* 105: 337-344.

Shadin, I., G. Magdoleen, Osman and M.K. Mutasim. 2009. Effect of growth regulators, explant and genotype on shoot regeneration in Tomato (*Lycopersicon esculentum* cv. Omdurman). *Int. J. Sustain. Crop Prod.,* 4(6): 7-13.

Sharma, M., A. Kothari-Chajer, Jagga-Chugh and S.L. Kothari. 2011. Factors influencing *Agrobacterium tumefaciens*-mediated genetic transformation of *Eleusine coracana* (L.) Gaertn. *Plant Cell Tiss. Org. Cult.,* 105: 93-104.

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

Sheeja, T., E.A. Mondal and R.K. Rathore. 2004. Efficient plantlet regeneration in tomato (*Lycopersicon esculentum* Mill.). *Plant Tissue Cult*., 14: 45-53.

Shilpa, K.S., V.D. Kumar and M. Sujatha. 2010. *Agrobacterium*-mediated genetic transformation of safflower (*Carthamus tinctorius* L.). *Plant Cell Tiss. Org. Cult*., 103: 387-401.

Shivakumar, B., J.B. Mythilli, L. Anand and G.V.S. Saiprasad. 2007. Influence of genotype on *Agrobacterium*-mediated transformation of tomato. *Indian J. Hort*., 64: 251-257.

Svabova, L. and M. Griga. 2008. The effect of co-cultivation treatments on transformation efficiency in pea (*Pisum sativum* L.). *Plant Cell, Tiss. and Org. Cult*., 95: 293-304.

Tian, L., K.S. Jing, H.L. Zhao and L. Qing. 2011. Transformation of HBsAg (Hepatitis B Surface Antigen) gene into Tomato mediated by *Agrobacterium tumefaciens*. *Czech J. Genet. Plant Breed*., 47(2): 69-77.

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

Vidya, C.S.S., M. Manoharan, C.T.R. Kumar, H.S. Savithri and G.L. Sita. 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.

Wang, B., L.J. Liu, X.X. Wang, J.Y. Yang, Z.X. Sun, N. Zhang, S.M. Gao, X.L. Xing and D.X. Peng. 2009. Transgenic ramie [*Boehmeria nivea* (L.) Gaud.]: factors affecting the efficiency of *Agrobacterium tumefaciens* mediated transformation and regeneration. *Plant Cell Rep*., 28: 1319-1327.

Wu, H.W., T.A. Yu, J.A.J. Raja, H.C. Wang and S.D. Yeh. 2009. Generation of transgenic oriental melon resistant to Zucchini yellow mosaic virus by an improved cotyledon-cutting method. *Plant Cell Rep.,* 28: 1053-1064.

Yasmin, A. and T. Debener. 2010. Transient gene expression in rose petals via *Agrobacterium* infiltration. *Plant Cell Tiss. Org. Cult*., 102: 245-250.

Ye, Li, H.X. and G.L. Zhou. 1994. *In vitro* culture of tomato cotyledons and regenerated plants. *J. Huazhong Agric. Uni*., 13: 291-295.

Zambre, M., N. Terryn, J. De Clercq, S. De Buck, W. Dillen, M. Van Montagu, D. Van Der Straeten and G. Angenon. 2003. Light strongly promotes gene transfer from *Agrobacterium tumefaciens* to plant cells. *Planta*, 216: 580-586.

Zelcer, A., O. Soferman and S. Izhar. 1984. An *In vitro* screening for tomato genotypes exhibiting efficient shoot regeneration. *J Plant Physiol.,* 115: 211-215.

(Received for publication 7 November 2014)