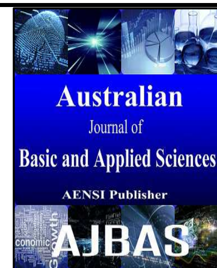
Australian Journal of Basic and Applied Sciences, 9(33) October 2015, Pages: 411-416

ISSN:1991-8178

Australian Journal of Basic and Applied Sciences

Journal home page: www.ajbasweb.com



An efficient and reproducible regeneration and transformation protocol in tomato (*Solanum lycopersicum* L.)

Reda E.A. Moghaieb, Dalia S. Ahmed, Abdel-Hadi A. Abdel-Hadi and Ahmed N. Sharaf

Department of Genetics and Genetic Engineering Research Center. Faculty of Agriculture, Cairo University, P.O. 12613- Giza, Egypt.

ARTICLE INFO

Article history:
Received 3 October 2015
Accepted 31 October 2015

Keywords:
Tomato, regeneration, transformation, PCR, gene expression

ABSTRACT

Background: Tomato (*Solanum lycopersicum*) is an important vegetable crop of *Solanaceae*, and is considered as an important human nutritional source containing minerals, vitamins and amino acids. In Egypt tomato is subjected to several a biotic and biotech stresses that affect its productivity. **Objectives:** Establishment of an efficient *in vitro* regeneration protocol is an important prerequisite to utilize the advantage of cell and tissue culture for genetic improvement**.** In the present study the regeneration and transformation efficiency from hypocotyl explants of five tomato cultivars was examined. **Results:** The data indicated that embryonic calli were formed within two weeks in the presence of 1 mg/l 2,4-D. Adventitious shoots emerged from the embryonic calli in the presence of 2 mg/l BA. The cultivars showed a varied response to shoot regeneration. The cultivar Promodora exhibited higher regeneration frequency (48%) followed by Castle Rock (47%) then by Super strain B (36%) compared with the other cultivars tested. Hypocotyl explants from the cultivars Castle Rock and Super strain B were inoculated and co-cultivated with *Agrobacterium tumefaciens* strain LBA4404 harboring a binary vector *pBI-121* containing the neomycin phosphotransferase-II gene (*npt-II*). The resulted putative transgenic plantlets were able to grow under knanamycin containing medium. The stable integration of the *npt-II* gene into the plant genomes was confirmed by PCR using *npt-II* -specific primers. The expression of *gus* gene was detected by histochemical GUS assay method which indicates that the expression was found only in the transgenic plants. **Conclusion:** The reported protocol in the present study is repeatable and can be used to regenerate transgenic tomato plants expressing the genes present in *A. tumefaciens* binary vectors.

© 2015 AENSI Publisher All rights reserved.

To Cite This Article: Reda E.A. Moghaieb, Dalia S. Ahmed, Abdel-Hadi A. Abdel-Hadi and Ahmed N. Sharaf., An efficient and reproducible regeneration and transformation protocol in tomato (*Solanum lycopersicum* L.). ***Aust. J. Basic & Appl. Sci.,*** *9(33): 411-416, 2015*

INTRODUCTION

Tomato (*Solanum lycopersicum* L. formerly known as *Lycopersicon esculentum* Mill) (2n =2x =24) is commercially important vegetable crop throughout the world. It is grown under a wide range of climates (temperate or tropical) in the open field or under protected cultivation. Tomatoes are very valuable for human health since they are low in fat and calories, free of cholesterol and rich in vitamins A and C, β-carotene, lycopene and potassium, as well as octadecadienoic acid (Kim *et al.,* 2011).

Tomato is subjected to some destructive diseases among which are wilt and root rots, early and late blights caused by Fusarium, Rhizoctonia, Alternaria and Phytophthora, respectively. Fusarium wilt of tomato caused by *Fusarium oxysporum* ((Schlecht.) f. sp. lycopersici (Sacc.)), is one of the most prevalent, serious diseases of tomato (Reis *et al.*, 2005 and Sudhamoy *et al.,* 2009).

There is a great potential for genetic manipulation in tomato to enhance productivity through increasing pest and disease resistance, environmental stress tolerance and to study gene function and regulation. Establishment of an efficient *in vitro* regeneration protocol is an essential prerequisite for harnessing the advantage of cell and tissue culture for genetic improvement. A transfer of regeneration capacity from *Lycopersicon peruvianum* to *L. esculentum* was successfully achieved by Wijbrandi *et al.,* (1988), and the genes controlling shoot regeneration in tomato were characterized and mapped by Koornneef *et al.*, (1993).

To date several methods for *in vitro* regeneration of tomato have been described and cotyledons are the choice explant as they are quickly established and possess a high morphogenetic potential (Kaur and Bansal, 2010). However limitations with tomato regeneration system includes, morphogenetic response is highly growth regulator-dependent

Corresponding Author: Reda E.A. Moghaieb, Department of Genetics and Genetic Engineering Research Center. Faculty of Agriculture, Cairo University,P.O. 12613- Giza, Egypt.

(Bhatia *et al.,* 2004), genotype specific response (Park *et al.,* 2003) and in most cases regeneration of shoots has been obtained through callus (Singh *et al.,* 2010; Kaur and Bansal, 2010).

Transformation in tomato and other dicotyledonous plants via *A. tumifacience* is still far from routine and there is no universal procedure suitable to transform different cultivars within each species. Dicotyledonous species differ widely in their organogenic potential and amenability to genetic transformation. Most techniques for genetic transformation and regeneration depend on the use of plant growth regulators is complex and nearly empirical combinations adapted to each particular situation (Romero *et al.,* 2001).

Although tomato transformation *via* Agrobacterium through adventitious shoot regeneration is not considered a real problem, the interest to obtain more efficient, reliable, simple and universal methods for tomato transformation is appreciated.

Since tomato regeneration is genotype and procedure dependent, standardization of regeneration protocol for desired genotype is very important for genetic transformation. The aim of this study was to develop a procedure for the transformation and regeneration of viable shoots from hypocotyl explants of commercial Egyptian tomato cultivars. Five commercial cultivars of tomato were successfully regenerated. Differences in the regeneration capacity among the cultivars were observed. The system described here can be used as a basis for the future development of commercial scale production of regenerated tomato plants. In addition, the co transformation of a foreign gene with T-DNA in the Ti plasmid of *A. tumefaciens* leads to an improvement of plant properties.

MATERIALS AND METHODS

Plant material:

Five commercial tomato cultivars namely, Promodora, Castle Rock, Super strain B, Flora Dade and Red star were used in the present study.

Seed sterilization:

Seeds were surface sterilized by dipping in 70% ethanol for 1 min, followed by immersion in 30% sodium hypochlorite for 15 min, and three rinses in sterile distilled water. Seeds were blot dried on sterilized Whatman filter papers. The sterilized seeds were germinated in petri dishes containing 0.8% (w/v) agar. The plates were sealed with parafilm and incubated at 25°C under a 16/8 hrs light/ dark condition.

Callus induction medium:

Hypocotyl (3 mm in length) explants were isolated from one week old seedlings. Explants were cultured in modified MS medium (Murashige and Skoog 1962), contained MS salt, 3% (w/v) sucrose, B5 vitamins, 1% (w/v) agar and was supplemented with 1.0 mg /l 2,4-D , (pH 5.8). Each plate contained 10 explants and the treatments were performed with 10 replicates. The plates were sealed with parafilm and incubated at 25°C under a 16/8 h light/dark condition. The explants were sub-cultured weekly on corresponding medium freshly prepared.

Shoot induction:

Embryonic calli were transferred into shoot induction medium that contains MS salts supplemented with 2 mg/l BA. The culture was incubated at at 25°C under a 16/8 h light/dark condition. Data were collected from three independent experiments on the following traits: callus induction and shoot induction frequency that was calculated as follows: Callus induction frequency = (number of calli producing explants divided by the total number of explants) X 100; shoot induction frequency = (number of shoots producing explants divided by the total number of explants) X 100. The analysis of variance was carried out according to the methods described by Snedecor and Cochran (1956).

Shoot elongation and acclimatization:

Shoot initiation (1-2-cm lengths) was transferred to growth regulator free MS medium. The regenerated plantlets were transferred to sterilized vermiculite in 15 cm plastic pots and irrigated with Hoagland solution (Hoagland and Arnon 1950), in a humid chamber at 25°C, under a 16/8 h day/night cycle. After acclimatization for three weeks, the plants were grown under green house conditions. The plant regeneration frequency was calculated based on the (number of plants producing shoots divided by the total number of shoots initiated in the culture) X 100.

Production of gus expressing transgenic plants:

Tomato seeds (cv. 'Castle Rock and cv. Super strain B) were germinated on MS medium. Hypocotyl explants were excised from 6-day-old seedlings and used for transformation.

Co-cultivation:

A. tumefaciens strain LBA4404 harboring the binary *Ti vector pBI-121*(Jefferson *et al*. 1987) were grown overnight in 30 ml of LB medium containing 50 µg/ml kanamycin sulfate, and then collected by centrifugation at 1120 x g for 5 min. The pellet was re-suspended in MS medium containing 100 µM acetosyringon. The hypocotyl explants prepared from 6-day-old seedlings were immersed in the bacterial suspension for 5 min. The explants were then blotted with sterilized filter paper and placed on a co-cultivation medium consisting of MS medium with 100 µM acetosyringon and supplemented with 2 mg/l

2,4,D. After co-cultivation for three days the explants were transferred to the shoot induction medium consisting of MS salts supplemented with 2 mg/l BA. Three weeks later, adventitious shoots emerged from the cut ends of the explants. T_0 plantlets that had regenerated *in vitro* and could survive under kanamycin containing medium were transferred to pots and grown to maturity in a greenhouse. T_1 seeds obtained by self-pollination of T_0 plants were surface-sterilized and germinated on MS medium containing 50 mg/l kanamycin. The germinated seedlings were transferred to a greenhouse and used for further analysis.

PCR analysis:

In order to confirm the stable integration of the T-DNA into the plant genome, the putative *gus* expressing plantlets were analyzed by PCR using *npt-II* gene specific primers. DNA samples were isolated from transformed plantlets according to the method described by Rogers and Bendich (1985). The reaction mixture (20 µl) contained 10 ng DNA, 200 µM dNTPs, 1 µM of each primer, 0.5 units of Red Hot Taq polymerase (ABgene House, UK) and 10-X *Taq* polymerase buffer (AB gene Housse, UK). Samples were heated to 94°C for 5 min, then subjected to 35 cycles of 1 min at 94°C; 1 min at 56°C and 1 min at 72°C. The PCR products were separated by agarose gel electrophoresis and visualized with ethidium bromide. The primers for the *npt-II* gene were designed for the positions 24-49 and 254-277, the sequences were 5`CGCAGGTTCTCCGGC CGCTTGG GTGG-3` and 5`-GACTTCGCCTTCCCTGA CCGACGA-3.

Histochemical GUS assay:

To screen for the expression of β-glucurodinase (GUS) in transformed tomato plants histochemical GUS assay was carried out according to the method of Jefferson *et al*., (1987). For analysis, plant tissues were incubated in a reaction buffer containing 12.5 mM $K_3Fe\ (CN)_6$, 12.5 mM $K_4Fe\ (CN)_6$, 20% methanol, 1% Triton X-100 and 38.3 mM 5-bromo-4-chloro-3-indolyl glucuronide as a substrate for the enzyme. The tissue was incubated in staining solution at 37°C for 24hrs and the developed blue spots were recorded.

RESULTS AND DISCUSSION

In order to induce embryogenic calli hypocotyl explants that isolated from five tomato cultivars were cultured on MS medium supplemented with 1 mg/l 2,4-D. The explants exhibited an initial swelling followed by callus formation within two weeks of incubation. It was noted that callus proliferation started from cut ends of the hypocotyls on MS medium supplemented with 1 .0 mg/l 2,4-D. In general, a high percentage of explants formed callus (40-86%, Table 1). Significant differences in callus induction between the cultivars were observed (Table 3). One week later the somatic embryos directly originated from the callus (Fig.1). Callus induction efficiency among different tomato cultivars used is presented in Table (1). The data indicated that the cultivar Promodora showed higher callus induction ability compared with the other cultivars (Table 1).

Table 1: Callus induction percentages among five tomato cultivars cultivated in Ms medium supplemented with 2 mg/l 2,4-D

Varieties	No. of explants	No. of calli forming explants	Callus induction %
Promodora	600	520	86
Castle Rock	600	450	75
Super Strain B	600	300	50
Flora dade	600	280	46
Red Star	600	240	40

Table 2: Regeneration capacity differences among five tomato cultivars

Varieties	No. of calli forming S.E.	Total No. of shoot initiations	No. of regenerated plants	Regeneration %
Promodora	400	800	390	48
Castle Rock	300	650	310	47
Super Strain B	180	180	65	36
Flora dade	100	100	25	25
Red Star	90	70	15	21

Data in Table (2) indicate that BA promoted shoot induction in all the cultivars, and the Promodora cultivar consistently showed the highest number of shoots per explant, followed by Castle Rock, Super strain B, Flora Dade and by Red star. Table (2) showed that, the cultivars differed in the number of regenerated plants. The cultivar Promodora gave the highest number of regenerated plants followed by Castle Rock, Super strain B, Flora Dade and Red star. This finding supports the assumption that regeneration in tomato is genotype-specific. These data are consistent with the findings reported by Chen and Adachi (1994), Ali and Li (1994), and Lindhout *et al.,* (1987).

Hypocotyl explants-derived calli were placed on regenerating media containing 2 mg/l BA. The nodular structures developed into shoot buds when the embryogenic calli were sub-cultured in the medium supplemented with BA within two weeks. The somatic embryos directly emerged from the

body of the explants or indirectly germinated from the embryonic callus (Figure 1). Figure (1- B-F) depicts the different developmental stages of the plantlets. The data in Figure 2 indicate that exogenous BA promoted shoot induction in all the cultivars, and the cultivars Promodora and Castle Rock showed higher capability for production of somatic embryos at 1mg/l BA compared with the other cultivars used. The initiated shoots were matured and germinated on half strength MS medium. The seedlings were acclimatized and transferred to the greenhouse. Table (2) shows that the regeneration frequency was 48, 47, 36, 25 and 21% for the cultivars Promodora, Castle Rock, Super strain B, Flora dade, and Red star respectively. The number of shoots per explant varied significantly between the five cultivars. The regeneration frequency was 48, 47, 36, 25 and 21% for the cultivars Promodora, Castle Rock Super Strain B Flora dade and Red Star respectively (Table 2). The data collected from three experiments for both callus and shoot induction were pooled together and were statistically analyzed by the combined analysis of variance according to the method described by Snedecor and Cochran (1956).

Data in Table (3) indicate that the difference between cultivars in callus induction was highly significant. The observed differences in shoot induction between cultivars in response to BA were anticipated due to the genetic differences between them. These results were in agreement with those of Keyes *et al.,* (1980) and Kageyama *et al.,* (1988) who reported that plant regeneration and somatic embryogenesis are genotype dependent. In several reports the influence of the genetic background on morphogenesis and embryogenesis in vitro was emphasized (Rapela 1985; Shillito *et al.,* 1989; Chu *et al.,* 1990. According to Kageyama *et al.,* (1988), in tomato the ability of callus to form shoots was genotype specific.

Table 3: Analysis of variance showing the differences between the tomato cultivars in response to callus and shoot formation

S.V	d.f	Callus induction (MS)	Shoot induction (MS)	$F_{0.05}$
Cultivars	4	6701.7**	4797.6 **	3.83
Replicate	2	437.20 $^{n.s}$	170.53 $^{n.s}$	4.46
Error	8	419.47	342.30	
Total	14	---	---	

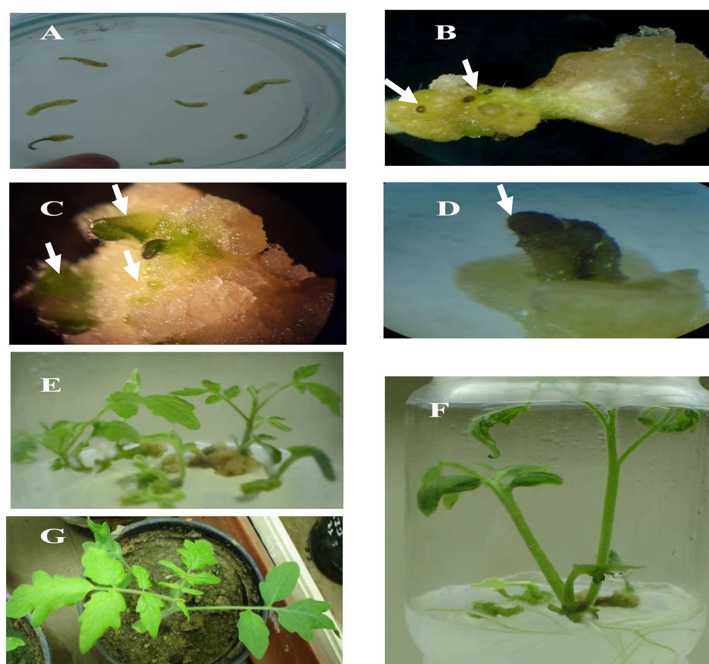


Fig. 1: different steps of whole plant regeneration from the hypocotyl explants. A: starting explants, B-D callus induction and somatic embryos development (arrows indicate the different developmental stages of somatic embryos), E: shoot regeneration, F: rooting of regenerated shoots and G: acclimatization process

Different factor were found to play major roles in tomato transformation: Agrobacterium cell density (Velcheva *et al.,* 2004), regeneration and co-cultivation conditions (Drorri and Altman, 2001), acetosyringone and cell competence after wounding and bacterial strain (Drorri and Altman 2001). All these studies indicated that the regeneration and transformation of the tomato is highly dependent on

the genotype and needs to be established for each cultivar (Velcheva *et al.,* 2004, Shahriari *et al.,* 2006).

The regeneration protocol of the present study was efficiently used to produce transgenic tomato plants expressing the *npt-II* gene. Hypocotyl explants from the cultivars Castle Rock and Super Strain –B were co-cultivated with *A. tumefaciens* for 3 days. After co-cultivation, the infected hypocotyls were placed on MS medium with low selection pressure. Under these conditions, the ends of the hypocotyls gradually initiated the formation of callus. During the process of selection, the successfully transformed hypocotyls continued to grow vigorously to produce calli, whereas the untransformed ones failed to form callus and eventually bleached and became necrotic within 3 weeks. Shoots were usually regenerated within 4 to 6 weeks on the MS medium after co-cultivation. During the selection culture, sub-culturing the explants with a change of fresh medium containing 50 mg/l kanamycin greatly reduced the number of escapes. The data indicated that the transformation percentages was 5.97 and 4.06 for the cultivar Castle Rock and Super Strain –B respectively (Table 4).

Table 4: The transformation percentage of tomato cultivars co-cultivated with Agrobacterium

Genotype	No. of explants used	No. of shoot initiation	$Kana^R$ plants	PCR positive for *npt-II*	No. of *gus* expressing plants	Transfor-mation %
Castle Rock	300	820	49	49	49	5.97
Super Strain -B	300	787	32	32	32	4.06

To confirm the presence of the *T-DNA* in the regenerated plants, all T_0 plants were subjected to PCR analysis with the primers specific for the *npt-II* gene. The PCR analysis indicated that all the regenerated plants examined showed a clear band corresponding to the relevant sequence of *npt-II* gene (250 bp) (Figures 2-B). In the present study the kanamycin resistant plants showed *gus* expression while the non-transgenic did not show any GUS activity (Figure 2-C).

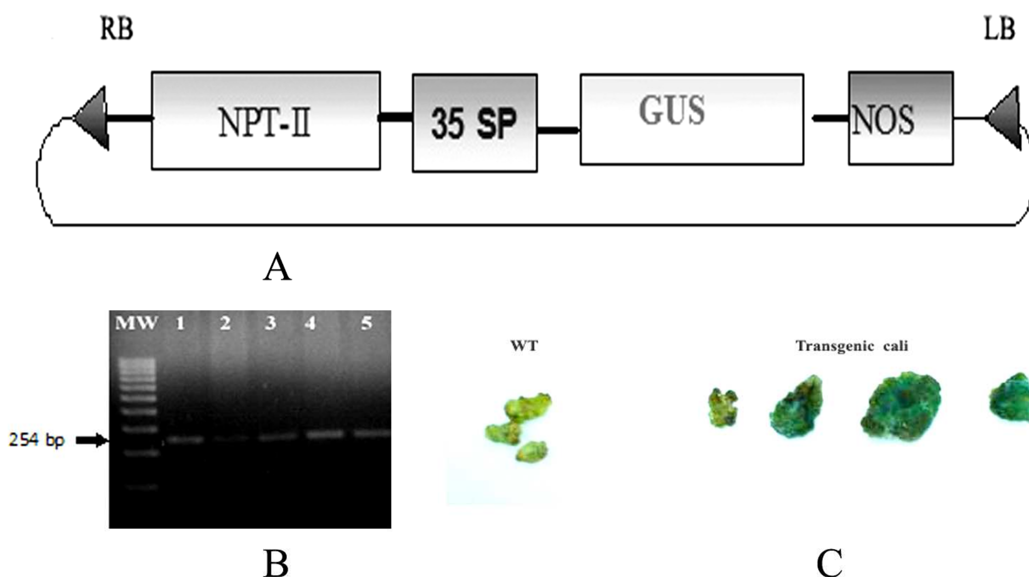


Fig. 2: (A) Showing the schematic representation of the T-DNA region containing the *gus* gene under the control of the 35 S promoter. (B) PCR confirmation of the stable integration of the *npt-II* gene into plant genome and (C) indicating the *gus* gene expression in non-transgenic (WT) and transgenic calli.

Based on the data of the present study we can conclude that the reported regeneration system is repeatable and can be easily used to regenerate transgenic tomatoes expressing the genes present in the *Agrobacterium* binary vector T-DNA. Using this regeneration and transformation protocol we can achieve our main goal which is the production of fungal resistant transgenic tomato plants.

REFERENCES

Ali, Y. and S.J. Li, 1994. Effect of various growth regulators on the initiation, size, color and differentiation of callus in different genotypes of tomato. Sarhad J. Agric., 10: 399-405.

Bhatia, P., A. Nanjappa, S. Tissa and M. David, 2004. Tissue culture studies in tomato (*Lycopersicon esculentum*). Plant Cell Tissue Organ Cult., 78: 1-21.

Chen, L.Z. and Adachi, T. 1994: Plant regeneration via somatic embryogenesis from cotyledon protoplasts of tomato (*Lycopersicon esculentum* Mill.). Breeding Sci., 44: 257-262 .

Chu, E.E., R.D. Hill and B. Bable, 1990. High frequency of pollen embryo formation and plant regeneration in *Triticum aestivum* L.: monosaccharide containing media. Plant Sci., 121: 112-122.

Drorri, E. and A. Altman, 2001. Transformation of tomato with the *βA* gene to confer osmotic stress tolerance (glycine-betaine production). Plant Sci., 49: 152-153.

Jefferson, R.A., T.A. Kavanagh, M.W. Bevan, 1 987. GUS fusion. βglucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J. 6: 3901-3907.

Kageyama, K., K. Yabe and S. Miyajima, 1988. Plant regeneration from cotyledon of tomato (*Lycopersicon esculentum* Mill.). Res. Bull. Aichi-Ken Agric. Res. Cent., 20: 167-171.

Kaur, P. and K.C. Bansal, 2010. Efficient production of transgenic tomatoes via Agrobacterium- mediated transformation. Biol. Planta., 54(2): 344-348.

Keyes, G.J., G.B. Collins and N.L. Taylor, 1980. Genetic variation in tissue. Culture of red clover. Theor. Appl. Genet., 5: 265-271.

Kim, Y.I., S. Hirai, H. Takahashi, T. Goto, C. Ohyane and T. Tsugane, 2011. 9-Oxo- 10(E), 12(E)-octadecadienoic acid derived from tomato is a potent PPAR a agonist to decrease triglyceride accumulation in mouse primary hepatocytes. Mol. Nutr. Food Res., 55: 585-593.

Koornneef, M., J. Bade, C. Hanhart, K. Horsman, J. Schel, W. Soppe, R. Verkerk and P. Zabel, 1993. Characterization and mapping of gene controlling shoot regeneration in tomato. Plant J., 3: 131-141.

Lindhout, P., R. Karsten and A. Doesum, 1987. Regeneration from leafdiscs of 100 genotypes of tomato. Acta Bot. Neerl., 36: 331-335.

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. PhysioL Plant, 15: 473-479.

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.

Rapela, M.A., 1985. Organogenesis and somatic embryogenesis in tissue cultures of Argentine maize (*Zea mays* L.). J. Plant Physio., 121: 119-122.

Reis, A., H. Costa, L.S. Boiteux and C.A. Lopes, 2005. First report of *Fusarium oxysporum* f. sp. *lycopersici* race 3 on tomato in Brazil. Fitopatologia Brasileira, 30: 426-428.

Rogers, S.O., A.J. Bendich, 1985. Extraction of DNA from milligram amounts of fresh herbarium and mummified plant tissues. Plant Mol. Biol., 5: 69-76.

Shahriari, F., H. Hashemi and B. Hosseini, 2006. Factors influencing regeneration and genetic transformation of three elite cultivars of tomato (*Lycopersicon esculentum* L.). Pak. J. Biol. Sci., 9: 2729-2733. DOI: 10.3923/pjbs.2006.2729.2733.

Shillito, R.D., C.M. Johanson, J.J. DiMaio and C.T. Hrams, 1989. Regeneration of fertile plants from protoplasts of elite inbred maize. Biotechnology, 7: 581-587.

Singh, A., M. Singh and B.D. Singh, 2010. Comparative *in vitro* shoot organogenesis and plantlet regeneration in tomato genotypes. Indian J. Hort., 67(1): 37-42.

Snedecor, G.W. and W.G. Cochran, 19560. Statistical Methods, 5th ed, The Iowa State University Press, Ames, Iowa

Romero, P.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 Tissue Org., 67: 173-180.

Sudhamoy, M., M. Nitupama and M. Adinpunya, 2009. Salicylic acid induced resistance to *Fusarium oxysporum* f. sp. *Lycopersici* in tomato. Plant Physiol. Biochem., 47: 642-649.

Velcheva, M., Z. Faltin, M. Flaishman, Y. Eshdat and A. Perl, 2005. A liquid culture system for *Agrobacterium*-mediated transformation of tomato (*Lycopersicon esculentum* L. Mill.). Plant Sci., 168: 121-130.

Wijbrandi, J., J.G.M. Vos and M. Koornneef, 1988. Transfer of regeneration capacity from *Lycopersicon peruvianum* to *L. esculentum* by protoplast fusion. Plant Cell Tissue Organ Cult., 12: 193-196.