Research Article

Agrobacterium Mediated Genetic Transformation of Tomato using *Cry2A* Gene

K. Deepa[1], N. Balakrishnan[2], Ameena Premnath[3], S. Jeyarajan Nelson[1], D. Sudhakar[2] and V. Udayasuriyan[2]*

[1]Department of Agricultural Entomology, Tamil Nadu Agricultural University, Coimbatore - 641 003, Tamil Nadu, India
[2]Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore - 641 003, Tamil Nadu, India
[3]Department of Oil Seeds, Tamil Nadu Agricultural University, Coimbatore - 641 003, Tamil Nadu, India

Abstract

Tomato (*Solanum lycopersicum* L.) is an important vegetable crop cultivated throughout India. The crop is extensively damaged by a lepidopteran insect, *Helicoverpa armigera* Hubner. To mitigate this problem, an attempt was made to generate transgenic tomato plants resistant to fruit borer. In this study, cotyledonary explants of cv. Arka Vikas were transformed with *cry2A* gene encoding insecticidal crystal protein of *Bacillus thuringiensis* through *Agrobacterium* mediated transformation. Transformation studies resulted in generation of 26 plants with regeneration efficiency of 4.86 per cent. Out of the 26 regenerated plants, nine were established in greenhouse. Screening by PCR revealed the presence of *cry2A* gene in six out of nine putative transformants and 1.12 per cent transformation efficiency was recorded. None of the six PCR positive transformants showed detectable level of Cry2A protein expression.

Keywords: Tomato transformation, *cry2A* gene, *Helicoverpa armigera*

***Correspondence**
Author: V. Udayasuriyan
Email:udayvar@yahoo.com

Introduction

Cultivated tomato (*Solanum lycopersicum* L.) is one of the important member of the family *Solanaceae*, which also includes potato, pepper, tobacco and many other plants [1]. It is a second most important *Solanaceous* vegetable crop next to potato in the world [2]. In India, it is cultivated in an area of 7.9 lakh ha with 17.3 M mt production [3]. Tomato is extensively damaged by the lepidopteran insect *Helicoverpa armigera* Hubner, also called tomato fruit borer. Farmers apply numerous insecticidal sprays to manage the pest as it has developed resistance to insecticides [4]. Moreover deployment of these agrochemicals has resulted environmental and health hazards.

Cultivation of insect resistant varieties is the most economical and effective approach to protect crops from insect attack [5]. A wild species of tomato *viz., Lycopersicon hirsutum* f. *glaburatum* has been reported to be highly resistant to fruit borer [6]. Screening of 44 tomato accessions/ varieties for resistance to *H. armigera* has revealed low to moderate level of resistance [7]. However, transfer of the resistance gene through conventional breeding is time consuming, resource and labour intensive [2]. Genetic engineering technology has helped plant researchers to transfer genes from irrelevant origin into significantly important crop plants to induce insect resistance [8]. Tomato has relatively small DNA content and is a genetic model for crop improvement [9]. Earlier in 2003, *Agrobacterium* mediated transformation of tomato cotyledonary explants was optimized with bacterial suspension in exponential growth [10]. The transformation studies in tomato were done earlier by many researchers for lycopene [11], β-carotein [12], and other *cry* genes [13]. *Bacillus thuringiensis* (Bt), a gram-positive soil bacterium, produces crystalline inclusions during sporulation, which contain insecticidal proteins called δ-endotoxins. Most strains of *Bt* are specifically toxic to lepidopteran insects. The commercial *Bt* formulations are effective against 50 lepidopteran insect species [14]. Transgenic tomato plants developed by using *cry1Ab* gene [15, 16] and *cry2Ab* gene [17] were reported to be resistant to lepidopteran insect pests such as *H. armigera, S. litura* and *Pthorimoaea operculella.* In the present investigation, an attempt was made to develop transgenic tomato plants harbouring *cry2A* gene through *Agrobacterium* mediated transformation.

Materials and Methods

Agrobacterium strain and plant transformation vector

In the present study, *Agrobacterium tumefaciens* strain LBA4404, harbouring binary vector p2AT with *cry2A* gene

obtained from Prof. V. Udayasuriyan, Department of Plant Biotechnology, Tamil Nadu Agricultural University, was used. This vector also has *nptII* gene as a plant selectable marker gene, conferring resistance to kanamycin.

Plant material and growth conditions

Seeds of tomato cv. Arka Vikas obtained from IIHR, Bangalore was used in this study. Tomato seeds were washed with sterile distilled water for three times followed by ethanol (70%) wash for five minutes and surface sterilized with 4 per cent sodium hypochlorite solution containing two to three drops of Tween 20 for five to seven minutes. Then the seeds were rinsed with sterile distilled water for two to three times followed by air drying on sterile tissue paper. The sterilized seeds were germinated on half strength MS medium [18] under 48 hours dark period followed by 8/16 dark light cycle. A tissue culture room or growth chamber maintained at 25±1°C was used for seed germination and maintenance of pre- culture, co- cultivation and plant regeneration. From the ten day old seedlings, cotyledons were cut at the tip and base (to a size of approximately $0.5cm^2$) and placed on pre- culture medium (adaxial surface in contact with the medium) for one day. Explants were handled gently with sterile flat forceps to avoid injury.

Agrobacterium mediated transformation of tomato

The *Agrobacterium* strain harbouring plant expression constructs grown on YEP medium at 28°C shaker and 200 rpm were harvested by centrifugation at 6,000 rpm for 10 min. The pellet was washed with 10 ml of MS broth and suspended in MS broth containing 100 mM acetosyringone. Density of the *Agrobacterium* in the suspension was maintained at 0.1OD at 600 nm and used for cocultivation. The pre cultured explants were carefully submerged in the suspension (30 ml of co-cultivation suspension) in a sterile petri plate for 15 minutes. After the exposure, explants were blotted on sterile tissue paper and transferred to co-cultivation medium. Plates were kept under dark in a growth chamber for 48 hours. After the co-cultivation period, the explants were washed with a washing medium and blot dried. Later the co-cultivated explants were transferred to shoot regeneration medium containing kanamycin for the selection and maintained under 8/16 dark light cycle. The explants that responded well were sub cultured on to the fresh shoot regeneration medium for three times at every 15 days interval. The well developed shoots were transferred to rooting medium. After profused rooting plants were hardened in a pot mixture containing sterile sand: soil: vermicompost in 1:1:1 proportion and covered with polythene cover for seven days in a culture room, and transferred to green house. The regeneration efficiency was calculated by using the following formula,

$$\text{Regeneration frequency} = \frac{\text{Number of plants regenerated}}{\text{Number of explants cocultivated}} \text{X } 100$$

Molecular and biochemical analysis of putative transgenics

Genomic DNA was extracted from leaves of putative transformants and non transformed control plants. A small leaf (50 to 100 mg) was ground in a 2.0 ml Eppendorf tube containing 300 µl CTAB buffer (100 mM Tris-HCl pH 7.5, 1.4 M NaCl, 20 mM EDTA and 2 % CTAB) and was incubated in a water bath at 65ºC for 1 hr. Then equal volume of choloroform: isoamyl alcohol @ 24:1 ratio was added and centrifuged at 12,000 rpm for 10 minutes after mixing by inversion. The supernatant was collected and to this, an equal volume of ice cold isopropanol was added and kept at -20ºC for 1 hr. The DNA was pelleted by centrifugation at 12,000 rpm for 5 minutes. The pellet was washed briefly with 70 per cent ethanol and air dried at room temperature. The pellet was dissolved in 30 µl of 0.1X TE buffer (1mM Tris- HCl pH 8.0 and 0.1mM EDTA pH 8.0). PCR was performed with the genomic DNA isolated from putative transformants to confirm the presence of *cry2A* and *npt*II genes using gene specific primers. Reaction with 25µl volume was performed using 100 ng of genomic DNA, 2.5 µl of 10X PCR buffer, 75 mM dNTPs, 50 ng of forward and reverse primers, 1.5 U Taq DNA polymerase. These primers amplify 1183 and 712 bp internal fragments of *cry2A* and *npt*II respectively. DNA isolated from non-transformed control plants were used as control and the reaction mix without template DNA were used as negative control. The transformation efficiency was calculated by using the following formula,

$$\text{Transformation efficiency} = \frac{\text{Number of PCR positive transgenic events}}{\text{Number of explants cocultivated}} \text{X } 100$$

Qualitative analysis of expression of Cry2A protein in putative transgenic tomato plants was carried out using commercially available Envirologix Cry2A kit by following the manufacturer’s instructions. This ELISA kit is a

sandwich type *i.e*. wells in the microtitre plate is coated with antibodies against test protein. Protein in the sample binds to antibodies and then gets detected by the enzyme (horse radish peroxidase) labelled antibody. The positive and negative controls provided along with the kit were used as reference. Enzyme conjugate (50 µl) was added to each well of the Cry2A antibody pre-coated plate. Approximately 20 mg of leaf tissue was homogenized in 250 µl of extraction buffer, mixture was spun at 6000 rpm in 4ºC for 7 minutes and supernatant was immediately used for assay. Wells were covered with parafilm and incubated at ambient temperature for 45 minutes. After 45 minutes, wells were thoroughly washed with 1X wash buffer. About 100 µl substrate was added to each well and the parafilm covered microtitre plate was incubated for 15 minutes. Then the reaction was stopped by adding 100 µl of the stop solution. The wells containing positive samples turn yellow. Optical density (O.D.) of the plate was read at 450 nm using negative control as blank. Each sample was replicated twice and each well was considered as one replication.

Results

Regeneration and transformation efficiency

The cotyledonary explants co-cultivated with *Agrobacterium* strain LBA4404 harbouring (p2AT) for 48hrs recorded 4.86 per cent (**Table 1**) regeneration efficiency on kanamycin selection (**Figure 1**). Regenerated putative transformants were analysed for confirmation of transgene *cry2A* and *npt*II gene by PCR. Six out of nine putative transformants showed the presence of transgene *cry2A* and *npt*II gene (**Figure 2**). The transformation efficiency under kanamycin selection was found to be 1.12 per cent (Table 1).

Biochemical analysis of putative transformants

In order to find out the expression of *cry2A* gene in the six PCR positive transformants, qualitative ELISA was performed using antibodies specific to Cry2A protein. The six plants which were found to be positive for the presence of *cry2A* gene and for the presence of *nptII* gene showed negative results for the expression of *cry2A* gene.

Table 1 *Agrobacterium* mediated transformation of tomato using cotyledonary explants

Name of the construct used	No. of explants co-cultivated	No. of shoots regenerated	Regeneration efficiency (%)	No. of plants established in green house	No. of PCR positive plants	Transformation efficiency (%)
p2AT	534	26	4.86	9	6	1.12

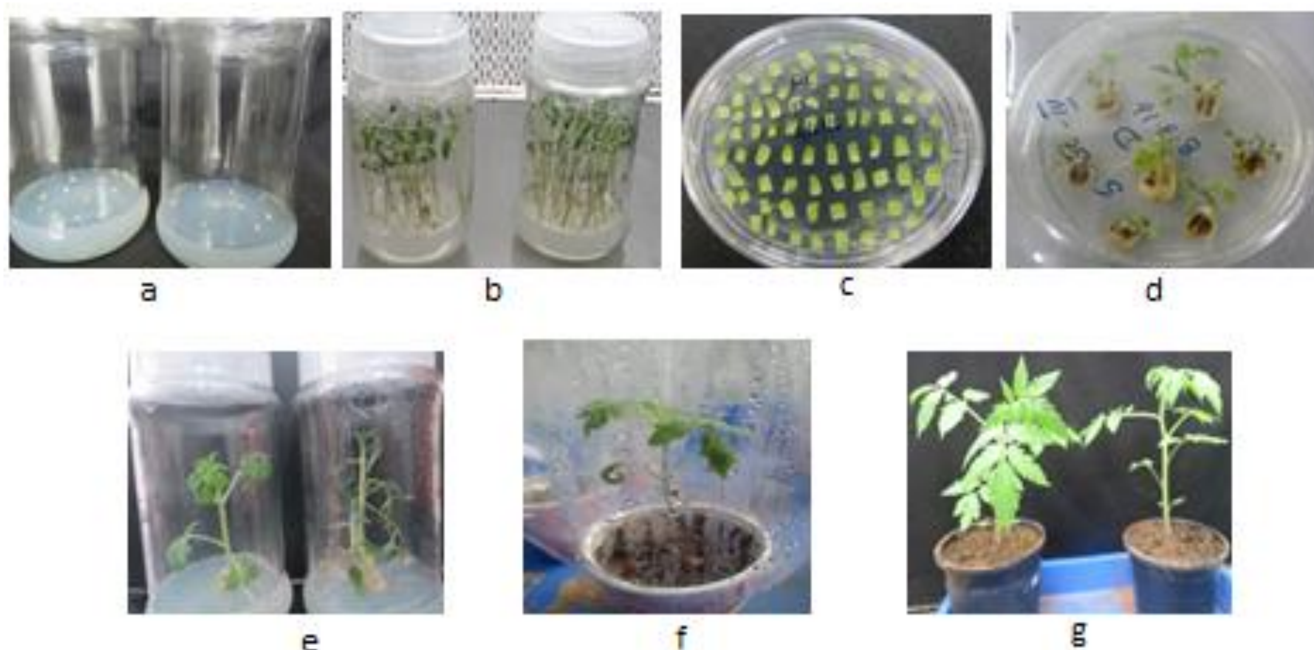

Figure1 *Agrobacterium* mediated transformation of tomato. (a) Seed inoculation on half MS medium, (b) Seedlings for explant- cotyledon, (c) Explant inoculated with *Agrobacterium*, (d) Callus initiation from inoculated explant, (e) Regenerated putative transformants on rooting medium, (f) Hardened putative transformants of tomato in greenhouse, (g) Putative transformants of tomato transferred to pots

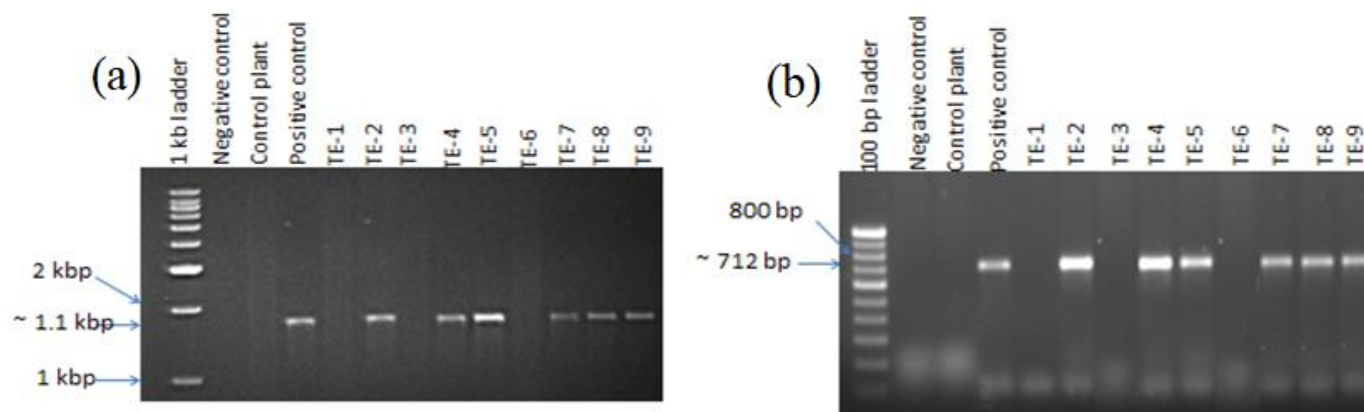

Figure 2 Molecular analysis of putative transformants of tomato. (a& b) 1.1 kbp and 712 bp internal sequence of *cry2A* and *nptII* gene respectively, amplified by PCR from the DNA isolated from putative transformants of tomato

Discussion

Insect resistant crops were developed through *Agrobacterium* mediated transformation of *cry* genes from Bt that produces crystal proteins toxic to certain insects belonging to the order Lepidoptera, Diptera and Coleoptera [19, 20]. In the present study, *cry2A* gene was introduced to tomato cultivar Arka Vikas and screened by PCR and ELISA. Arka Vikas performs very well in the Indian subcontinent and has been developed from an American variety Tip Top by pure line selection [21]. In 2014, transgenic tomato plants were developed using truncated Bt-*cry1Ab* gene through *Agrobacterium*-mediated leaf-disc transformation procedure [16]. It had been also, reported that young explants show better morphogenic response than the older ones [22]. Accordingly, cotyledonary explants obtained from eight day old seedlings of tomato cv. Arka Vikas were used in the present study. The hypocotyl explants for regeneration and transformation of tomato with *cry2A* gene through Agrobacterium- mediated protocol were attempted by different authors [23, 24]. However, in earlier studies, [25] cotyledonary explants showed organogenesis superiority over hypocotyls and leaf explants. Most published protocols for tomato transformation [26, 10] describe co-cultivation of the explants with various *Agrobacterium* strains (LBA4404, C58C1, GV311SE or A208) for 48 hours with variable densities. In the present study, co-cultivation upto 48hrs using *A. tumefaciens* strain, LBA4404 harbouring p2AT construct recorded 4.86 percent regeneration efficiency. Nine putative transgenic plants were developed in the present study. Among them, six plants were found to be positive for the presence of *cry2A* and *nptII* gene and recorded a transformation efficiency of 1.12 percent which is very low when compared to the results obtained by the other earlier workers. In tomato, transformation frequencies show variation among cultivars. Transformation frequencies have ranged from 6-49 per cent [27, 28]. Efficiency of transformation is affected by many factors like *Agrobacterium* strain, inoculation duration, co-cultivation time, preselection period, concentration of acetosyringone or other antibiotics in the media, type of explants and genotype [29]. Transformation efficiency values reported in the literature are 8% [26], 7–37% [30], 9% [31], 11% [32], 20% [33, 27], 25% [34] and 28–48% [35]. The PCR positive transformants were subjected to qualitative ELISA to determine the expression of Cry2A protein. None of the six PCR positive transformants showed positive results for the expression of *cry2A* gene. This leads to the conclusion, that there are certain obstacles related to the expression of transgene such as intactness of the gene or its site of integration.

References

[1] C. Janani, S. Girija, R.B.D. Kumari, *In vitro* culture and *Agrobacterium* mediated transformation in high altitude tomato (*Lycopersicon esculentum* Mill.) cultivar Shalimar. *International Journal of Pharmacy Teaching and Practices*., 2013, 4, p 483-488.

[2] M.R. Fooland, Genetics of salt and cold tolerance in tomato: quantitative analysis and QTL mapping. *Plant Biotechnology*., 2007, 16, p 55-64.

[3] National Horticulture Board, Indian Horticulture Database, Gurgoan, 2015. http://www.nhb.gov.in.

[4] Anonymous, *Heliothis* management. In: Jayaraj, S., Uthamasamy, S., Gopalan, M., Rabindra, A.R.J. (eds.). Proceedings of the National Workshop. Centre for Plant Protection Studies. Tamil Nadu Agricultural University, Coimbatore, India, 1990.

[5] C. Wu, Y. Fan, C. Zhang, N. Olica, S.K. Datta, Transgenic fertile japonica rice plants expressing a modified *cry1A (b)* gene resistant to yellow stem borer. *Plant Cell Reports*, 1997, 17, p 129-132.

[6] H. Salinas, C.F.A. Vallejo, S.E. Estrada, Evaluation of resistance to the tomato fruit borer *Neolenncinodes elegagantalis* (Guenee) in material of *Lycopersicon hirsutum* Hubm and Bopnl and *L. pimpinelliform* (Just) Mill. and its transfer to cultivated material of tomato *L. esculentum*. *Ade-Agron-Uni- Nacl-de Colombia*., 1993, 43, p 1–4.

[7] M. Amutha, S. Manisegaran. Screening of tomato accessions/varieties against *Helicoverpa armigera*. *Annals of Plant Protection Science*., 2006, 14 (1), p 239-240.

[8] G.W. Lycett, D. Grierson, Genetic engineering of crop plants. *Butterworth Heinemann*, London, UK. 1990. p.303.ISBN.0-40-804779-8.

[9] K. Arumuganathan, E.D. Earle, Nuclear DNA content of some important plant species. *Plant Molecular Biology Reporter*., 1991, 9, p 208–218.

[10] P. Ellul, B. G. Sogo, B. Pineda, G. Rios, L. A. Roig, V. Moreno, The ploidy level of transgenic plants in *Agrobacterium* mediated transformation of tomato cotyledons (*Lycopersicon esculentum* L. Mill.) is genotype and procedure dependent. *Theoretical and Applied Genetics*., 2003, 106, p 231-238.

[11] P.D. Fraser, E.M.A. Enfissi, J.M. Halket, M.R. Truesdale, D.M. Yu, C. Gerrish, P.M. Bramley, Manipulation of phytoene levels in tomato fruit: effects on isoprenoids, plastids, and intermediary metabolism. *Plant Cell*., 2007, 19, p 3194–3211.

[12] S. Romer, Elevation of the provitamin A content of transgenic tomato plant. *Nature Biotechnology*., 2000, 18, p 666-669.

[13] H. Paramesh, B. Fakrudin, M.S Kuruvinashetti, Genetic transformation of a local variety of tomato using *gus* gene: an efficient genetic transformation protocol for tomato. *Journal of Agricultural Technology*., 2010, 6(1), p 87-97.

[14] D. Fischhoff, A, K. S. Bowdish, F. J. Perlak, P. G. Marrone, S. M. McCormick, J. G. Niedermeyer, D. A. Dean, K. Kusano-Kretzmer, E. J. Mayer, D. E. Rochester, S. G. Rogers, R. T. Fraley, Insect tolerant transgenic tomato plants. *Bio Technology*., 1987, 5, p 807-813.

[15] H. Kumar, V. Kumar, Tomato expressing Cry1A(b) insecticidal protein from *Bacillus thuringiensis* protected against tomato fruit borer, *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae) damage in the laboratory, greenhouse and field. *Crop Protection*., 2004, 23(2), p 135-139.

[16] B. Koul, S. Srivastava, I. Sanyal, B. Tripathi, V. Sharma, D.V. Amla, Transgenic tomato line expressing modified *Bacillus thuringiensis cry1Ab* gene showing complete resistance to two lepidopteran pests. *Springer Plus*., 2014, 3, p 84.

[17] M. Saker, H. Salama, M. Salama, N.M. Abd El-Ghany, Production of transgenic tomato plants expressing *cry2Ab* gene for the control of some lepidopterous insects endemic in Egypt. *Journal of Genetic Engineering and Biotechnology*., 2011, 9, p 149–155.

[18] T. Murashige, F. A. Skoog, A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Plant Physiology*, 1962, 15, p 473-497.

[19] R. Chitkowski, L, S. G. Turnipseed, M. J. Sullivan, W. G. Bridges, Field and laboratory evaluations of transgenic cottons expressing one or two *Bacillus thuringiensis* var. *kurstaki* Berliner proteins for management of Noctuidae (Lepidoptera) pests. *Journal of Economic Entomology*., 2003, 96, p 755-762.

[20] K. Bashir, T. Husnain, T. Fatima, N. Riaz, R. Makhdoom, S. Riazuddin, Novel indica basmati line (B-370) expressing two unrelated genes of *Bacillus thuringiensis* is highly resistant to two lepidopteran insects in the field. *Crop Protection*., 2005, 24, p 870-879.

[21] K. Lopez, E. Libas, S. Shanmugasundaram, Vegetable research networking in South Asia; Savernet phase I final report (Shanhua, Tainan, Taiwan: AVRDC). 1996. p 76

[22] M. C. Harish, S. Rajeevkumar, R. Sathishkumar, Efficient *in vitro* callus induction and regeneration of different tomato cultivars of India. *Asian Journal of Biotechnology*., 2010, 3, p 178-184.

[23] S. Vageeshbabu, K. Hanur, K.N. Boopal, S. Srividya, S.B. Poovarasan, M.S Saraswathi, V.A. Vijeth, P.V. Rami Reddy, Development of c*ry2A* Bt transgenic tomato for resistance to the tomato fruit borer, *Helicoverpa armigera*. In Tumkur University and CSIR sponsored National Level Conference on “Dissecting the Complexities of Plant Biotechnology in the Post-Genomic Era”, 21st September, 2011, PP. 16- 32.

[24] V. S. Hanur, B. Reddy, V. V. Arya, P. V. R. Reddy, Genetic transformation of tomato using Bt *cry2A* gene and characterization in Indian cultivar Arka Vikas. *Journal of Agricultural Science and Technology*., 2015, 17, p 1805-1814.

[25] P. Mamidala, R. S. Nanna, Effect of genotype, explants source and medium on *in vitro* regeneration of tomato. *International Journal of Genetics and Molecular Biology*., 2011, 3, p 45-50.

[26] C.S. Vidya, M. Manoharan, C.T.R. Kumar, H.S. Savithri, G.L. Sita, *Agrobacterium* mediated transformation of tomato (*Lycopersicon esculentum* var. Pusa Ruby) with coat-protein gene of physalis mottle TYMO virus. *Journal of Plant Physiology*., 2000, 156, p 106 - 110.
[27] D. Qiu, G. Diretto, R. Tavarza, G. Giuliano, Improved protocol for *Agrobacterium* mediated transformation of tomato and production of transgenic plants containing carotenoid biosynthetic gene *CsZCD*. *Scientia Horticulturae*., 2007, 112, p 172-175.
[28] N. Jabeen, B. Mirza, Z. Chaudhary, H. Rashid, M. Gulfraz, Study of the factors affecting *Agrobacterium* mediated gene transformation in tomato (*Lycopersicon esculentum* Mill.) cv. Riogrande using rice chitinase (*cht-3*) gene. *Pakistan Journal of Botany*., 2009, 41, p 2605-2614.
[29] N. Ahsan, S. H. Lee, D. G. Lee, M. Anisuzzaman, M. F. Alam, H. S. Yoon, M. S. Choi, J. K. Yang, B. H. Lee, The effects of wounding type, preculture, infection method and cocultivation temperature on the *Agrobacterium* mediated gene transfer in tomatoes. *Annals of Applied Biology*., 2007, 151(3), p 363-372.
[30] H.Q. Ling, D. Kriseleit, M. D. Ganan, Effect of tricarcillin / potassium clavulanate on callus growth and shoot regeneration in *Agrobacterium* mediated transformation of tomato (*Lycopersicon esculentum*). *Plant Cell Reports*., 1998, 17, p 843-847.
[31] J.S.C.V. Roekel, B. Damm, L.S. Melchers, A. Hoekema, Factors influencing transformation frequency of tomato (*Lycopersicon esculentum*). *Plant Cell Reports*., 1993, 12: p 644- 647.
[32] A. Frary, E. Earle, An examination of factors affecting the efficiency of *Agrobacterium* mediated transformation of tomato. *Plant Cell Reports*., 1996, 16, p 235- 240.
[33] S. H. Park, J. L. Morris, J. E. Park, K. D. Hirschi, R. H. Smith, Efficient and genotype independent *Agrobacterium* mediated tomato transformation. *Journal of Plant Physiology*., 2003, 160, p 1253-1257.
[34] W. Hu, G.C. Phillips, A combination of over growth-control and antibiotic improves *Agrobacterium tumefaciens* mediated transformation efficiency for cultivated tomato (*L. esculentum*). *In Vitro Cellular and Developmental Biology- Plant*., 2001, 37, p 12-18.
[35] H.J. Sun, S. Uchii, S. Watanabe, H. Ezura, A highly efficient transformation protocol for Micro-Tom, a model cultivar for tomato functional genomics. *Plant Cell Physiology*., 2006, 47, p 426–431.

© 2018, by the Authors. The articles published from this journal are distributed to the public under "**Creative Commons Attribution License**" (http://creative commons.org/licenses/by/3.0/). Therefore, upon proper citation of the original work, all the articles can be used without any restriction or can be distributed in any medium in any form.

Publication History
Received 15th Feb 2018
Revised 06th Mar 2018
Accepted 14th Mar 2018
Online 30th Mar 2018