International Journal of Pharmacy Teaching & Practices 2013, Vol.4, Issue 1, 483-488.

In vitro Culture and Agrobacterium Mediated Transformation in High Altitude Tomato (*Lycopersicon esculentum* Mill.) Cultivar Shalimar

C. Janani[1], S. Girija[2], B. D. Ranjitha Kumari[3*]

[1]Research Scholar and [3]Professor, Department of Plant Science, Bharathidasan University, Trichirapalli-24

[2]Assistant Professor, Department of Biotechnology, Bharathiyar University, Coimbatore

Research Article

Please cite this paper as: C. Janani[1], S. Girija[2], B. D. Ranjitha Kumari[3*]. *In vitro* Culture and Agrobacterium Mediated Transformation in High Altitude Tomato (*Lycopersicon esculentum* Mill.) Cultivar Shalimar. IJPTP, 2013, 4(1), 483-488.

Corresponding Author:

B. D. Ranjitha Kumari
Assistant Professor, Department of Biotechnology,
Bharathiyar University, Coimbatore
E-mail: ranithakumari2004@yahoo.co.in

Abstract

Tomato (*Lycopersicon esculentum* Mill.) belongs to the family Solanaceae. It is rich in vitamin A and C and also has the antioxidant properties. The *in vitro* response of high altitude tomato variety Shalimar was tested using the ten days old explants such as nodal and leaf for the multiple shoot formation and callus induction respectively. Among the different concentration of hormones tested, the maximum percentage (90%) of multiple shoot in MS medium containing BAP (2 mg/l). The callus induction (100%) was observed in 2, 4 –D (2 mg/l) combination with NAA (1 mg/l). Then the preliminary protocol for the efficient transformation of cv. Shalimar was standardized, using the binary vector pBI121 into the *Agrobacterium* strain LBA4404. And transient GUS expression was used as the basis for identifying the most appropriate conditions for transformation. The transformed leaf explants survived in selection medium (MS medium with 2 mg/l zeatin) fortified with 50 mg/l of each Kanamycin and Cefatoxime. The transient GUS gene was confirmed by which showed blue color formation through GUS assay.

Keywords: Lycopersicon esculentum · Agrobacterium · GUS · Kanamycin · Cefatoxime · 2, 4 –D · BAP · NAA · Zeatin.

Introduction

Tomato has been widely used not only as food, but also as research material. In addition, the tomato belongs to the family *Solanaceae* and is closely related to many commercially important plants such as potato, eggplant, peppers, tobacco and petunias. Knowledge obtained from studies conducted on tomato can be easily applied to these plants, which makes tomato as important research material. There are numerous studies have been reported on plant regeneration from a wide range of tissues and organs [1-2]. Adventious shoot regeneration is formed directly[3] and also form indirectly through the callus formation[4] and both the shoot and root formed together[5]. Response of shoot regeneration from the explants higher in the order of leaves, cotyledons and hypocotyls for the cell cultivars were proved[6]. Any part of plant species (preferably young explants) can be used to induce callus tissue. In culture this dedifferentiated mass of cells can be maintained indefinitely, provided the callus is sub cultured on fresh medium[7]. This study is used for the shoot formation and callus from the nodal and leaf explants of cv. Shalimar respectively in the MS medium. This is useful to develop the cv. Shalimar for the transformation to improve the quality of tomato for the high altitude people.

Genetic transformation is a strategy used to improve the quality and yield of tomato. Since the first report of *Agrobacterium* mediated tomato transformation[8], there have been many reports of genetic engineering in tomato for various purpose such as characterization of gene function, production of insect and disease resistant plants, herbicide tolerance, improved fruit quality, delay in fruit ripening, production of foreign proteins and improved transformation protocol[9-10]. Abiotic stresses including heat, salinity, drought and nutrient deficiencies often constrain fruit productivity[11-14]. Developing an effective approach for improving abiotic stress tolerance is essential[15]. Factors such as plant variety,[16] explant material and growth regulators[17] have an influence on the

efficiency of transformation in tomato. The present study it has been aimed to develop the protocol for transformation using *Agrobacterium* strain LBA4404 harboring pBI121 vector containing GUS reporter gene and kanamycin resistance *nptII* genes. This will be helpful for the improvement of the high altitude variety tomato in the nutritional level for the welfare of human health.

Material and Method

Plants

Tomato cv. Shalimar obtained from DIHAR, DRDO, Laddak, India (Fig 1).

***In vitro* studies**

The *in vivo* grown shoots were used as source of two types of explants: Nodal explants, leaf segments were cultured into tubes containing MS medium with different hormone concentration of BAP (0.5-2 mg/l) alone and the combination of 1 mg/l of IBA and KIN for multiple shoot formation and 2, 4 D (0.5-2 mg/l) individually and the combination of (0.5 and 1 mg/l) of NAA and IAA for callus induction.

***Agrobacterium* mediated transformation**

Agrobacterium tumefaciens strain LBA4404 was used for plant transformations. *Agrobacterium* used for co-cultivation carried the pBI121 vector. *Agrobacterium* cultures containing binary vectors were grown in shaking culture flasks for 72 hr at 28°C and 200 rpm. Cells were pelletted at room temperature at 5000 rpm, followed by washing with washing medium. The pellet was resuspended in 10 ml of washing medium. Bacterial cell density was measured using a spectrophotometer and adjusted to the final working concentration by diluting it with sterile liquid MS medium. The optical density (OD) 600 value of 0.6 corresponds to 10^8 cells/ml culture.

The growth room maintained at 25 ± 2°C was used for maintenance of pre culture, co cultivation and callus regeneration. *Agrobacterium* culture was carried out in a rotary shaker at 150 rpm maintained at 28°C. Leaf sections and shoots tips from matured tomato cv. Shalimar shoots were used. They were pre-cultured for 48 h at 28°C on pre-culture medium, with the adaxial surface in contact with the medium. Healthy explants that responded to pre-culture, as evident by swelling, were incubated in the bacterial suspension for 30 min and inverted every 10 min during incubation. The explants were then blotted on sterile tissue paper and co cultured on the same pre-culture medium for 72 hr at 28°C with 40-50 explants in bottles. After exposure for different experimental durations, co-cultured explants were washed 4–5 times with sterile MS liquid medium, blotted on sterile tissue paper and transferred to a selection medium containing 1 mg/L Zeatin for regeneration. Each petriplate (9 cm) had 20–25 explants for regeneration. Plates were cultured under a 16 hr light/8 hr dark cycle at 20°C. Explants that showed callus formation were sub cultured onto fresh selection medium every 15 days. The transformation efficiency was calculated as the per cent co cultivated explants producing independent transformation events, leading to regeneration of a complete plant on the selection medium.

Analysis of putative transgenic tomato explants

Histochemical assay was performed to visualize GUS activity[18]. Briefly, leaf tissues from randomly selected kanamycin resistant explants growing in the selection media were incubated in GUS histochemical buffer [50 mM sodium phosphate, pH 7.0; 50 mM EDTA, pH 8.0; 0.5 mM $K_3Fe(CN)_6$; 0.5 mM K_4 $Fe(CN)_6$; 0.1% Triton X-100; 1 mM X-gluc (5-bromo-4- chloro-3-indolyl β-D-glucuronide)] at 37°C for up to 24 hr. Chlorophyll in leaf tissues was subsequently extracted by incubation in acetone: ethanol (1:3) before assessment for GUS activity.

Results and Discussion

Tomato (*Lycopersicon esculentum* L.) is an economically important crop in many countries, including India. So various efforts were putforth by the researchers to improve the quality of this important crop towards disease resistance and improvement in their nutritional content. In the lime light of this the present results add further information to improve the crop through tissue culture and transformation based on the varietal differences in a response.

Multiple shoot initiation

The *in vitro* morphogenic responses of cultured plant tissues are affected by the different components of the culture media, especially by concentration of growth hormones, and it is therefore important to evaluate their effects on plant regeneration. Regeneration of tomato response depends largely on genotype, explants and plant growth regulators used in the medium. Response of shoot regeneration from the explants higher in the order of leaves, Cotyledons and hypocotyls for the cell cultivars were proved[6]. It was observed in 8 days old cotyledon producing higher number of shoots in zeatin supplemented media compared to BAP[19]. Multiple shoot regeneration of shoots was observed when cultured on MS medium supplemented with various concentrations (0.5 – 2 mg/l) of BAP alone and also in the combination of IBA and KIN (1 mg/l). Maximum number (10) of shoots were obtained at the concentration of BAP (2 mg/l) + IBA (1 mg/l) results were tabulated (Table 1) and formation of multiple shoot (Fig 2 and 3) were observed after 20 days of culture. The shoots were greenish showed continuous proliferation.

Callus Induction

The leaf explants cultured showed signs of callus induction within seven to ten days and calli were well developed within 20-30 days. In the present study, the excellent growth of callus was obtained in MS medium supplemented with 2, 4 –D (0.5 – 2 mg/l) individually and combined with NAA and IAA (0.5 and 1 mg/l) in Table 2, the mass of callus formation (Fig 4 and 5).

Transformation

Development of an efficient protocol for tomato transformation and its subsequent regeneration is a prerequisite for the production of transgenic plants. The shoot tip and leaf disc explants of Shalimar were co- cultivated with selected agrobacterium conjugant. Out of these, leaf disc explants, when placed on selection medium, induced callus within 3 weeks (Table 3). The shoot tip after transformation did not survive on the selection media (Fig 6-10).

Discussion

Multiple Shoot Initiation

Tomato is one of the most studied higher plants because of its importance as a crop species, and of several advantages for genetic, molecular and physiological studies[8]. Although previous reports on tomato in vitro regeneration studies showed different explants such as shoot, cotyledon, node regenerated shoots which were grown in MS medium[19]. The shoots were regenerated from excised hypocotyl segmants of mugwort on cytokinin - enriched medium and they report on the in vitro culture of *A. vulgaris* using TDZ, compare with BA[20]. The regeneration efficiency will differ from cultivar to cultivar, as it depends on the genotype of the explants used. So the studies carried out here to standardize the protocol for multiple shoot regeneration in this high altitude cultivar showed regeneration of multiple in MS medium supplemented with BAP and IBA and kinetin in contrast the previous studies on multiple shoot regeneration[19] where they used zeatin IAA for multiple shoot regeneration. Consequently the report on regeneration of shoots[21] showed the shoots were regenerated on MS medium supplemented with BAP + IAA and kinetin + IAA tomato hybrid plants met with callus induction and subsequent regeneration of plants. But the present study shows the standardized simple protocol for regeneration of shoots on MS medium supplemented with BAP and IBA and Kinetin and avoids using costly hormones like zeatin.

Callus Induction

The *in vitro* morphogenetic response of cultured plant tissues is effected by the different components of cultured media, especially by concentration of growth hormones, and it is therefore important to evaluate their effects on plant regeneration. In tomato, adventitious shoot regeneration can be achieved either directly or indirectly through and intermediate callus phase[22]. The combination of hormones used for callus induction is very important as the faith of callus weather to induce shoot regeneration or not also depends on it. Here in the present study the 2, 4-D induced calli can be further used for somatic embryogenesis in tomato.

Transformation

A simplified protocol to obtain transgenic tomato plants was established in the tomato cv. Micro-tom from leaf explants and it seems to be very useful for both micropropagation and genetic transformation purpose[23]. The transformation The transformation efficiency of tomato depends upon many factors such as the cultivar, type of explants and its age, *Agrobacterium* strain and its density, co cultivation time and regeneration medium[24]. Previous reports on tomato transformations suggest the use of a feeder layer during pre culture and *Agrobacterium* co cultivation[8] which makes the transformation procedure tedious. In our study, pre-culture and co cultivation were done on a basal medium of MS containing Zeatin 2mg/L in the absence of a feeder layer or acetosyringone. Most of the published protocols for tomato transformation describe co cultivation of the explants with various *Agrobacterium* strains for 48 h with bacterial densities ranging from 10^8 to 5.0 X 10^8 cells/ml, giving variable transformation efficiencies with different cultivars of tomato. In this study we observed that co cultivation for two days using bacterial strain LBA4404 at a density of 10^8 cells/ml enhanced the transformation efficiency. The infected explants were blotted dry using sterile filter paper and transfer to the co-cultivated medium for 3 days next the explants washed by MS liquid medium and transferred to the selection medium which contained cefotoxime to remove the bacterial growth and kanamycin for selection[25]. The removal of excess bacterial solution from the explants before transfer to the co-cultivation medium is also important. If the explants were blotted dry on tissue paper, bacterial growth didn't affect the health of explants. But if a little bacterial solution was left on the explants, bacterial growth affected the health of explants even within 48h.some explants showed a good level expression evenly where as some explants showed the GUS expression in the cut edges only. In our study we observed a high rate of survival (80%) of the transformed explants in the selection media. Out of the survived explants 10% showed callus induction. An investigation revealed that the efficiency of agrobacterium mediated transformation in safflower was showed a maximum frequency of 51.0% of putative transformants[26]. These results are indication of a good protocol. The further steps of the transformation such as shoot regeneration, rooting and hardening are also to be standardized for getting a complete transformation protocol for this variety of tomato. An simple and

efficient protocol for the production of sufficient numbers of transgenic tomato plants using reasonable number of explants from the Rio Grande tomato cultivar and report the transfer and expression of the vacuolar sodium antiporter and pyrophosphatase genes enhanced salt and drought tolerance in transgenic tomato plants[27]. Transformation of tomato with a bacterial codA gene enhances tolerance to salt and water stresses[28]. The Cry1Ac gene had been successfully introduced into the commercial variety 'Punjab Upma' and was useful for producing transgenic tomato resistant to fruit borer[29]. Likewise the present studied protocol is useful for the future experiments to use this optimized protocol for the production of transgenic tomato lines harbouring useful genes in this variety tomato.

TABLE 1: Effect of various concentrations of growth regulators used for multiple shoot from nodal explant of tomato (*Lycopercison esculentum*) cv. Shalimar.

S. No	Hormone (mg/L)	Percentage of response %	No. of shoots/explants	Shoot length (cm)
	BAP			
1	0.5	40	3.0±0.82	1.2±0.08
2	1	60	2.6±0.81	1.3±0.17
3	1.5	60	4.2±0.98	1.6±0.12
4	2	90	6.0±1.32	2.2±0.43
	BAP combination with IBA and KIN			
5	2 BAP + 1 IBA	60	7.6±1.21	2.9±0.12
6	2 BAP + 1 KIN	50	6.8±1.30	1.5±0.13

No. of shoot and the shoot length represented as Average ±

Standard deviation of shoot formation

TABLE 2: Effect of various concentrations of growth regulators on callus induction from leaf explants of tomato (*Lycopercison esculentum*) cv. Shalimar

S. No	Hormone concentration (mg/L)	Percentage of response (%)	Morphology of callus
I	2, 4-D		
1	0.5	50	Whitish friable
2	1.0	70	Brownish compact
3	1.5	80	Whitish friable
4	2.0	100	Greenish compact
II	2, 4-D combination with NAA and IAA		
5	2.0 2, 4-D +0.5 NAA		
6	2.0 2, 4-D +1.0 NAA	90	Whitish compact
7	2.0 2, 4-D +0.5 IAA	100	Whitish compact
8	2.0 2, 4-D + 1.0 IAA	70	Brownish compact
		90	Whitish compact

Efficient callus formation represented as (No. of regenerating explants/No. of plated explants) × 100.

TABLE 3: Percentage survival of transformed (co cultivated) and non-transformed (control) tomato explants at various stages.

Stage	Co-cultivated		Control	
	Survived	Percentage	Survived	Percentage
Co-cultivation	40/50	80	45/50	90
Callus initiation	5/50	10	25/50	50

Antibiotics (kanamycin 50mg/l and cefotaxime 50mg/l) were used for selection of transformant.

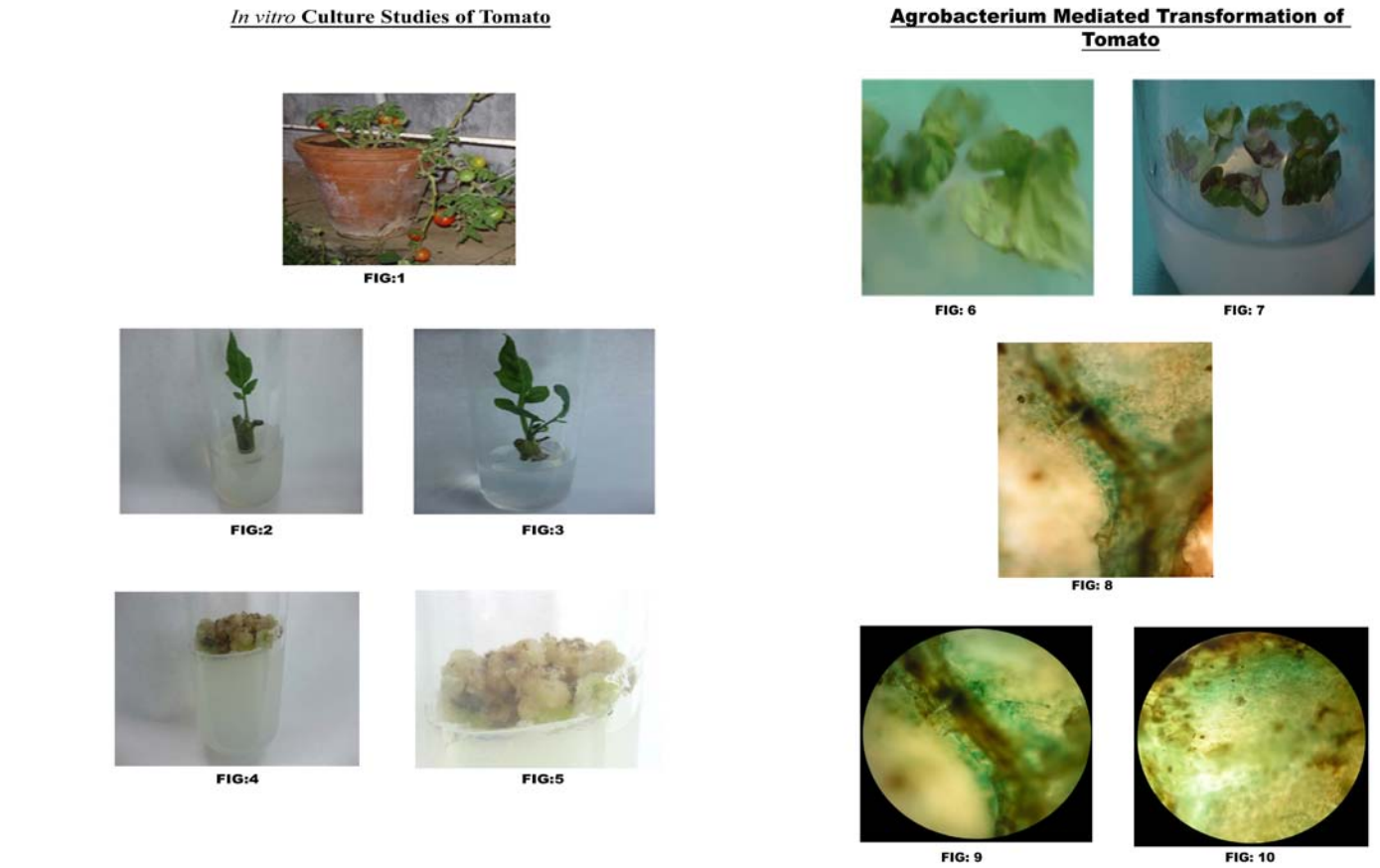

FIG 1: Tomato cv. Shalimar source of explant

FIG 2: Formation of shoots from the nodal explants of Tomato cv. Shalimar on MS medium supplemented with BAP 2mg/l.

FIG 3: Multiplication of shoot from the nodal explants of Tomato cv. Shalimar on MS medium supplemented with BAP 2mg/l.

FIG 4: Callus initiated from leaf explants of Tomato cv. Shalimar on the MS

FIG 5: Brownish compact callus induced from the leaf explants of Tomato cv. Shalimar on MS medium supplemented with 2, 4-D 2mg/l.

FIG 6: Pre culturing of explants

FIG 7: Explants in selection media (MS medium 2mg/l Zeatin)

FIG 8-10: GUS assay in section of transformed leaf (showed blue color)

References

1. Padmanaban VL, Paddock EF, Sharp WR. 1974. Plantlet formation from *in vitro* from *Lycopersicon esculentum* leaf callus. Can. J. Botany. 52: 1429-1432.
2. Novak FJ, Zadina J, Horackova V, Maskova I. 1980. The effect of growth regulators on meristem tip development and in vitro multiplication of *Solanum tuberosum L*. plants. Potato Res. 23: 155-166.
3. Dwivedi K, Srivastava P, Verma HN, Chatuvedi HC. 1990. Direct regeneration of shoots from leaf segments of tomato (*Lycopercison esculentum)* cultured *in vitro* and production of plants. Indian. J. Exp. Biol. 28: 32-35.
4. Behki RM, Lesley SM. 1980. Shoot regeneration from leaf callus of *Lycopersicon esculentum* (tomato). Can. J. Bot. 98: 83-87
5. Bhatia P. 2003. Optimization of physical, chemical and biological factors for *in vitro* micropropagation through direct regeneration, axillary branching and somatic embryogenesis method for 'red coat' cultivar of tomato (*Lycopersicon esculentum* mill.). Doctoral thesis. primary industries research centre, central queensland university, rockhampton, Australia.

6. Duzyaman E, Tanrisever A, Gunver G. 1994. Comparitive studies on regeneration of different tissues of tomato *in vitro*. Acta. Hort. (ISHS). 235-242
7. Giri A, Ahuja PS, Kumar PVA. 1993. Plant Cell Tiss Org 31: 213-218
8. McCormick S, Niedermeyer J, Fry J, Barnason A, Horsh R, Fraley R. 1986. Leaf disc transformation of cultivated tomato (*L. esculentum*) using *Agrobacterium tumefaciens.* Plant Cell Rep. 5: 81-84.
9. Fillati JJ, Kiser J, Rose R, Comai L. 1987. Efficient transfer of a glyphosate tolerance gene into tomato using a binary *Agrobacterium tumefaciens* vector. Nat. Biotech. 5: 726-731.
10. Janssen B, Lund L, Shinha N. 1998. Overexpression of homeobox gene, *Let6*, reveals indeterminate features in the tomato compound leaf. Plant Physiol. 117: 771-786
11. Raghothama KG. 2000. Phosphate transport and signaling. Curr. Opin. Plant. Biol. 3: 182-187.
12. Abel S, Ticconi CA, Delatorre CA. 2002. Phosphate sensing in higher plants. Physiol Plant 115: 1-8
13. Zhu J. K. 2002. Salt and drought stress signal transduction in plants. Annu. Rev. Plant Biol. 53: 247-273.
14. Bhatnagar-Mathur P, Vadez V, Sharma KK. 2008. Transgenic approaches for abiotic stress tolerance in plants: retrospect and prospects. Plant. Cell. Rep. 27: 411-424
15. Park S, Li J, Pittman JK, Berkowitz GA, Yang H, Undurraga S, Morris J, Hirschi KD, Gaxiola RA. 2005. Up-regulation of a H+-pyrophosphatase (H+-PPase) as a strategy to engineer drought-resistant crop plants. Proc Nat. Acad. Sci. U.S.A. 102: 18830–18835.
16. Ling HQ, Kriseleit D, Ganal MW. 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
17. Ohki S, Bigot C, Mousseau J. 1978. Analysis of shoot – formin capacity *in vitro* in two line capacity (*Lycopersicon esculentum*. Mill) and their hybrids. Plant Cell Physiol. 19: 27-42
18. Chaudhury A, Maheshwari SC, Tyagi AK. 1995. Transient expression of gus gene in intact seed embryos of indica rice after electroporation-mediated gene delivery; Plant. Cell. Rep. 14: 215–220.
19. Gubis J, Lajchova Z, Farag J, Jurekova Z. 2004. Effect of growth regulators on shoot induction and plant regeneration in tomato (*Lycopersicon esculentum* Mill.). Biologia- Bratislava-. 59/3: 405-408.
20. Sujatha G, Ranjitha Kumari BD. 2007. High- frequency shoot multiplication in Artimisia vulgaris L. using thidiazuron. Plant Biotechnol Rep. 1: 149-154
21. Shakeel AJ, Ghulam MS, Hidayat US, Rashid A. 2001. Differential *in vitro* response of tomato hybrids against a multitude of hormonal regimes. Online J. Bio. Sci. 12: 1141-1144.
22. Bhatia P, Ashwath N, Senaratna T, Midmore D. 2004. Tissue culture studies of tomato (*Lycopersicon esculentum*). Plant. Cell. Tiss Org 78: 1–21
23. Cruz-Mendivil A, Rivera-Lopez J, German–Baez JL, Lopez-Meyer M, Hernandez-Verdugo S, Lopez–Valenzuela JA, Vadez-Ortiz A. 2011. A simple and efficient protocol for regeneration and genetic transformation of tomato cv. Micro-tom from leaf explants. Hort. science 46.12: 1655-1600.
24. Sharma MK, Solanke AU, Jani D, Singh Y, Sharma AK. 2009. A simple andfieifent Agrobacterium - mediated procedure for transformation of tomato. J. Bioscience. 34(3): 423–433.
25. Seong ES, Ghimire BK, Goh EJ, Lim, JD, Kim, MJ, Chung, IM, Yu CY. 2009. Overexpression of the γ - TMT gene in *Codonopsis lanceolata*. Biol Plantarum. 53 (4): 631-636.
26. Shilpa SK, Dhinesh Kumar V, Sujatha M. 2010. Agrobacterium-mediated genetic transformation of safflower (Carthamus tintorius L.). Plant cell tiss organ cult. 103:387-401.
27. Khoudi H, Nouri-Khemaken A, Gouiaa S, Masmoudi K. 2009. Optimization of regeneration and transformation parameters in tomato and improvement of its salinity and drought tolerance. Afri J. Biotechnol. 8(22), 6068-6076
28. Goel D, Singh AK, Yadav V, Babbar SB, Murata N, Bansal KC. 2011. Transformation of tomato with a bacterial codA gene enhances tolerance to salt and water stresses. J. plant physiol 168.1286-1294.
29. Rashid R, Bal SS. 2011. Agrobacterium-mediated genetic transformation of tomato (Solanum lycopersicum L.) with Cry1Ac gene for resistance against fruit borer. J. tropical agricul. 49(1-2):110-113.

AUTHORS' CONTRIBUTIONS

Authors contributed equally to all aspects of the study.

PEER REVIEW

Not commissioned; externally peer reviewed.

CONFLICTS OF INTEREST

The authors declare that they have no competing interests.