Agrobacterium-Mediated Transformation of Tomato CV Arka Vikas with Cry2ax1 Gene for Insect Resistance

***[1]Bamishaiye E.I**, [1]Bala I., [2]Bamishaiye O.M., [3]Sudhakar D

[1]Perishable crop Research Department, Nigerian Stored Products Research Institute, Kano. Nigeria
[2]Dept of Biotechnology, Federal College of Agriculture Produce Technology, Kano. Nigeria
[3]Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India
***Corresponding author:** E.I. Bamishaiye, +2349034657480, bamishaiyeeunice@yahoo.com*

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Abstract
Tomato (Solanum lycopersicum) is one of the world's most preferred vegetable crops which is extensively damaged by lepidopteran insect pest, Helicoverpa armigera Hubner (tomato fruit borer). Insect pest causes serious damage in crop production, and various attempts have been
made to produce insect-resistant crops, including the expression of genes for proteins with
anti-herbivory activity, such as Bt (Bacillus thuringiensis) toxins. In order to mitigate this problem, an attempt was made to generate transgenic tomato plants resistant to fruit borer. Cotyledonary explants of tomato cv. Arka vikas were co-cultivated with Agrobacterium tumefaciens strain, LBA4404 harbouring a codon-optimised chimeric cry2AX1 gene driven by enhanced CaMV35S promoter in pCAMBIA2300 vector backbone. Seventy-six putative transgenic plants were regenerated, and the presence of the cry2AX1 gene in twenty eight plants was demonstrated by PCR analysis. ELISA showed that nine out of the twenty-eight plants had detectable level of Cry2AX1 protein expression, which ranged from 0.022 to 0.359µg/g of fresh tissue. Insect bioassay of transgenic T_0 tomato plants using H. armigera neonates recorded a mortality of 36.67 to 90 per cent and showed significant reduction in leaf feeding and inhibition of growth in surviving larvae. The results demonstrated the potential of the chimeric cry2AX1 gene in developing H. armigera resistant transgenic tomato varieties which can minimize the effect of climate change and decrease in beneficial soil flora through the use of synthetic chemicals.

Keyword; *Bacillus thuringiensis*; Cry2AX1; ELISA; *Helicoverpa armiger;*, Insect bioassay; transgenic;

1. Introduction

Tomato (*Solanum lycopersicum* L.) belongs to the Solanaceae family, and it is also a nutritionally important vegetable crop that is produced globally. It is cultivated as an annual crop which can successfully in both temperate and tropical conditions [1]. It is the world's largest vegetable crop after potato, and it tops the list of canned vegetables [2]. The cultivated tomato, *Solanum lycopersicon*, serves as a model plant species a well-studied crop species that is used for various research investigations including understanding of genetics, genomics, and breeding [3].

Genetic transformation is a vital process in plant molecular genetics and plant improvement. On a regular basis, agrobacterium-mediated transformation is used to introduce a foreign gene into the genome of plants, such as tomato [4, 5]. The insertion of a gene from *Bacillus thuringiensis* (*Bt*) that produces Cry proteins which are lethal to certain insect (*Lepidoptera*, *Coleoptera* and *Diptera*) families. These insecticidal crystal proteins selectively bind to insect midgut receptors and insert into the gut cell membrane, thereby causing the formation of pores in membrane leading to cell lysis and insect death [6]. Since the first transgenic tomato was developed about 30 years ago, significant improvement in the genetic transformation of tomato has been achieved in recent years [7].

One of the major limiting factors affecting tomato production worldwide, is the incidence of pests, often infesting or attacking at different stages of growth and development [8]. Some of the invading insect pests include tomato fruit borer (*Helicoverpa armigera*), jassid (*Amrasca biguttula biguttula*), whitefly (*Bemisia tabaci*), mite (*Tetranychus urticae*), aphid (*Myzus persicae*) and leaf miner (*Liriomyza trifolic*). Among all, tomato fruit borer, *Helicoverpa armigera* (Hub.) is an important lepidopteran pest responsible for severe yield loss which affects food security. Incidence and abundance of tomato fruit borer, *Helicoverpa armigera* (Hubner) is dependent on both the climatic parameters and the growth stage of the tomato crop. Pesticides application is an option and indiscriminate use, and misuse of pesticides results in adverse effects on the beneficial organisms, leading to resurgence and secondary outbreak of pest besides leaving residue in the edible fruit security [9].

Tomato engineered with different insecticidal protein gene(s) from *Bt* has been reported to provide adequate protection to plants against different lepidopteran insect pests [10, 11]. Due to the difference in structure and insecticidal activities, *cry2A* genes are suitable candidates for the management of insects in crop plants [12]. The chimeric Cry2AX1 protein was observed to be more lethal than its parental proteins (Cry2Aa and Cry2Ac) [13]. Considering these facts, the present study was conducted to develop transgenic tomato plants expressing *cry2AX1* gene, using *Agrobacterium* mediated transformation method and to evaluate the efficacy of *cry2AX1* expressed in tomato events against *H. armigera.*

2. Materials and Methods

2.1. Preparation of explants

Genetically pure seeds of tomato cv. Arka vikas were gotten from Horticultural College and Research Institute, Tamil Nadu Agricultural University, Periyakulam, Tamil Nadu. The seeds were soaked in water containing a few drops of tween 20 for five min and subjected to forceful shaking for another 5 min. Seeds were treated with seventy (70) per cent ethanol for 5 min and were washed with sterile water thrice. The seeds were further subjected to four (4) per cent sodium hypochlorite for 8 min with occasional whirling followed by doubled distilled water wash thrice. The seeds were blot dried on a sterile tissue paper and placed on half strength MS medium (Murashige and Skoog, 1962) for germination under sterile condition. The bottles were kept in the dark for a period of 72 hours followed by a cycle of 16 hours photoperiod using cool white fluorescent tube light (110-130 nM/m2/s intensity) and eight hours of darkness at 26˚C in a plant growth chamber (Panasonic, Japan). Cotyledonary explants were collected from 7 day-old *in vitro* grown seedlings (Figure 2A). The distal and proximal ends (1-2 mm) were cut off and the explants were cut into two pieces (Figure 2B) before placing them on the pre-culture medium (MS medium modified with Gamborg B5 vitamins containing 1 mg/L zeatin) [14]. They were handled gently with flat forceps to avoid any injury and were pre-cultured for a day under light prior to co-cultivation.

2.2. Binary vectors and strain

The pC2300-*2AX1* construct containing *cry2AX1* gene (Figure 1) was used in transformation experiments. The gene of interest was placed under the control of *EnCaMV35S* promoter and *nos-polyA* terminator in binary vector, pCAMBIA 2300 backbone. The vector which harbored the neomycin phosphotransferase (*nptII*) gene driven by CaMV35S promoter, conferring resistance to kanamycin which was used for plant selection. Independently, the construct was mobilized into *Agrobacterium* strain LBA4404 by triparental mating and the *Agrobacterium* transconjugants were grown on LB (1% Yeast extract, 1% Peptone and 0.5% NaCl pH 7.2) medium containing kanamycin 100 mg/L, tetracycline 5 mg/L and 10 mg/L rifampicin in a 28°C incubator shaker at 200 rpm for 48-72 hours and used for tomato transformation.

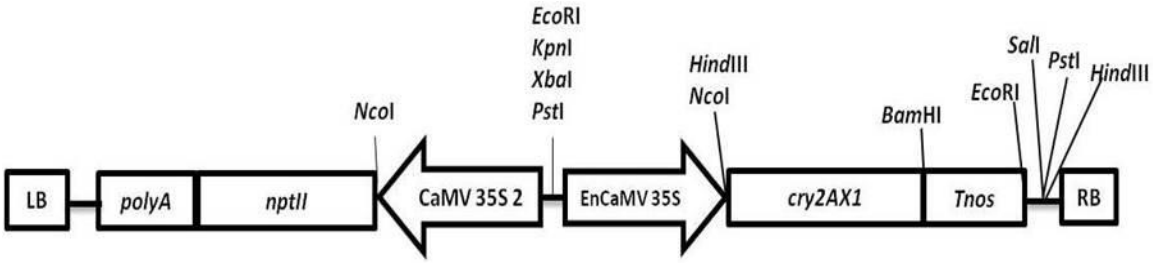


Figure 1. Schematic representations of T-DNA region of the plant transformation construct pC2300-*En35S-2AX1-Tnos*

LB: left border, poly A: CaMV35S terminator, *nptII*: neomycin phosphotransferase gene, CaMV35S2: Cauliflower mosaic virus 35S duplicated promoter, EnCaMV35S: Enhanced cauliflower mosaic virus 35S promoter, *cry2AX1*: gene of interest, *Tnos*: *nopaline synthase* terminator, RB: right border

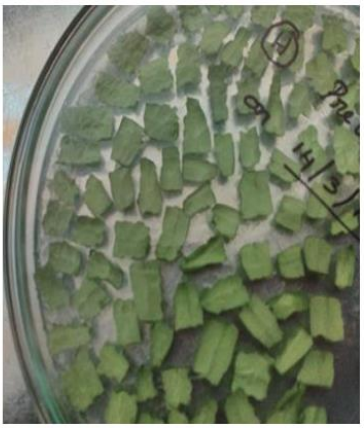
2.3. *Agrobacterium*-mediated transformation of tomato

A single colony of *Agrobacterium,* containing LBA4404 (pC2300-*2AX1)* gene was inoculated in sterile 3 mL LB medium (Yeast extract, Peptone and NaCl) containing kanamycin 100 mg/L, tetracycline 5 mg/L, and rifampicin 10 mg/ml, was allowed to grow overnight in an incubator shaker at 28°C and 180 rpm. From the overnight culture, an aliquot of 700 µl was inoculated into 30 ml LB with same antibiotics and conditions and grown for 6-8 hours. The bacterial pellets were harvested by centrifugation at 4,000 rpm for 10 min. The pellet was re-suspended in 40 ml of infection medium (modified MS containing 100 µM acetosyringone). *Agrobacterium* density in the suspension was maintained at 0.9 OD at 600 nm. The pre-cultured explants were carefully submerged in the suspension in a sterile petri plate for an infection time of 30 min with gentle agitation. Post infection, explants were blotted on sterile tissue paper and transferred onto co-cultivation medium (MS medium modified with Gamborg vitamins containing zeatin 1 mg/L and 100 µM acetosyringone). Plates were kept under dark condition in a growth chamber for 48 hours.

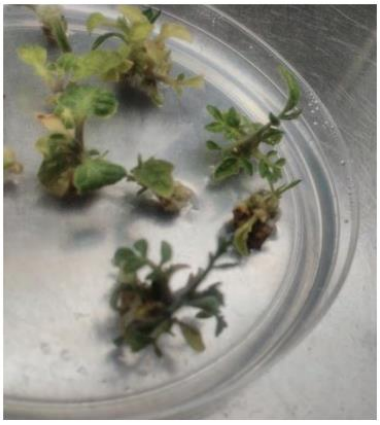
2.4. Regeneration of the transformed explants

After the co-cultivation period, the explants were washed in a washing medium (modified MS containing 200 mg/L timentin) to kill the *Agrobacterium*, blot dried and sub-cultured on selection medium (MS modified with Gamborg vitamins containing kanamycin 100 mg/L, zeatin 1 mg/L and 200 mg/L timentin) for shoot bud initiation and were maintained under 8/16 dark light cycle. The explants that responded well (Figure 2C) were sub-cultured continuously onto fresh medium, and later transferred to shoot elongation medium (MS modified with Gamborg vitamins containing kanamycin 100 mg/L, zeatin 0.5 mg/L and 200 mg/L timentin) for elongation. Elongated shoots (2-3 cm) (Figure 2D) were transferred to the half MS basic medium, supplemented with IBA (1 mg/L) for rooting and maintained under 16 hours light and 8 hours dark. Shoots with no sign of rooting after 21 days were discarded. Well rooted and established plants (Figure 2E) were hardened in transgenic greenhouse in small cups containing autoclaved coconut peat mixture covered with a polythene cover to maintain humidity. Well-established plants were transplanted into bigger pots and maintained in transgenic greenhouse (Figure 2F).

A) *In vitro* grown Arka vikas seedlings on germination medium



B) Arka vikas cotyledon on pre-culture medium



C) Co-cultivated A.V. on 3rd Selection medium

D) A.V. cotyledon on 4th selection medium

E) Elongated A.V shoot on rooting medium

F) Well established A.V transformant in transgenic greenhouse

2.5. Molecular characterization of transformants

2.5.1. DNA isolation and PCR analysis

Genomic DNA was isolated from leaves of putative transgenic and non-transgenic tomato plants using CTAB (cetyltrimethyl ammonium bromide) protocol, which is a modification of the method of [15]. Presence of transgene was confirmed by using *cry2AX1* gene specific primers (Forward 5'CCTAACATTGGTG GA CTTCCAG 3' and Reverse 5' GAGAAACGAGCTCCGTTATCGT 3') and *nptII* gene specific primers (forward primer 5'-AGAACTCGTCAAGAAGGCGA and reverse primer 5'-CAGACAATCG GCTGCTCTGA). The plasmid DNA was used as positive control. The PCR for both sets of samples was carried out in 50 µl reaction volume containing, 2.5 µl of 10X *Taq* buffer, 75 µM each of dNTPs, 50 ng each of forward and reverse primers and 1.5U of *Taq* DNA polymerase and incubated in a thermal cycler which was programmed for 5 min preheat at 94°C and then 35 cycles of denaturation at 94°C for 1 min, annealing temperature of 58°C for 45 sec and extension time of 45 sec at 72°C, with a final extension at 72°C for 7 min. The PCR products were run on 0.8 % agarose gel, visualized and documented in gel documentation system.

2.5.2. ELISA Analysis

A double-antibody sandwich Enzyme Linked Immuno Sorbent Assay (ELISA) was used to detect and quantify the Cry2AX1 protein expressed in the leaves of transgenic tomato plants. Cry2A quanti-plate (Envirologix, USA) ELISA kit was used for the experiment. Proteins from fresh leaf samples of transformed and untransformed tomato plants were extracted and used for detection of Cry2AX1 protein as per the manufacturer's protocol. The OD was measured at 450 nm using an ELISA reader (Biotek, USA). The quantity of Cry2AX1 protein present in the sample was calculated by referring to standard graph generated with Cry2A calibration standards and represented in µg/g fresh weight of leaves.

2.6. Insect Bioassay

Detached leaf bit bioassay was carried out to determine the level of insect resistance in ELISA positive T0 transgenic tomato plants with *H. armigera* neonates under laboratory condition. Leaf bits (1.4 cm diameter) from both the transgenic and control plants were placed in a damp filter paper on petri plates. Ten neonate larvae of *H. armigera* were released per replication and three replications were maintained in each line. The experiment was carried out at 27 ±1^{0}C and 65 per cent relative humidity. Larval mortality was recorded after 48 hours at 24 hours interval for six days.

Statistical analysis

The experimental data values of Cry2AX1 protein concentration and mortality of *H. armigera* were mean values from three replicates. All mortality data were subjected to arcsine transformations before analysis. Data analysis was done by analysis of variance (ANOVA) following the AGRES statistical package. Mean values were separated by Duncan's multiple range test (DMRT) at a 5 per cent probability level (Duncan 1955).

3.0. Result

3.1. Plant regeneration of co-cultivated explant

A total of seven six (76) putative transgenic tomato plants were regenerated out of five hundred and one (501) cotyledons that were co-cultivated C2300-2*AX1* construct. They are regarded as independent plant since they were derived from different co-cultivated explants.

PCR analysis

The seventy-six putative T_0 transgenic tomato plants generated were screened for the presence of *cry2AX1* gene and *nptII* gene with gene specific primers focusing on the coding region of *cry2AX1* and *nptII* gene, respectively. Figure 3a and 3b (representation) reveled twenty-eight transgenic plants generated with `pC2300-*2AX1* construct were found positive for PCR showing an amplification at the expected size of 430 bp fragment for *nptII* specific primers and 800 bp fragments for *cry2AX1* gene specific primers respectively. No amplification was recorded in non-transformed plants.

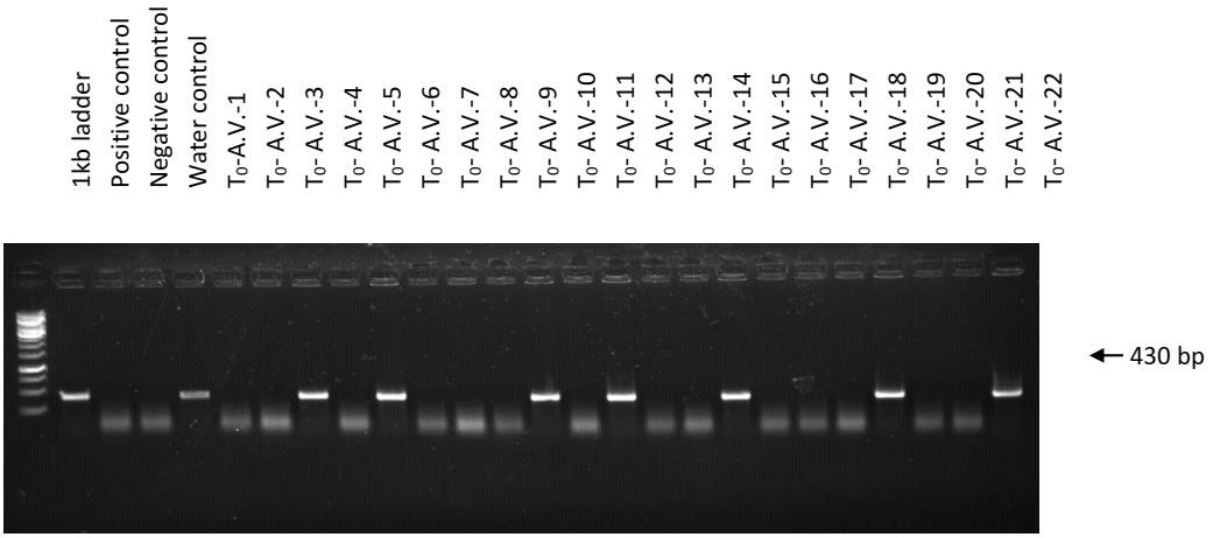


Fig 3a: PCR analysis of putative tomato *cv*. Arka Vikas transformants transformed with pC2300-*2AX1* for the presence of *nptII* gene

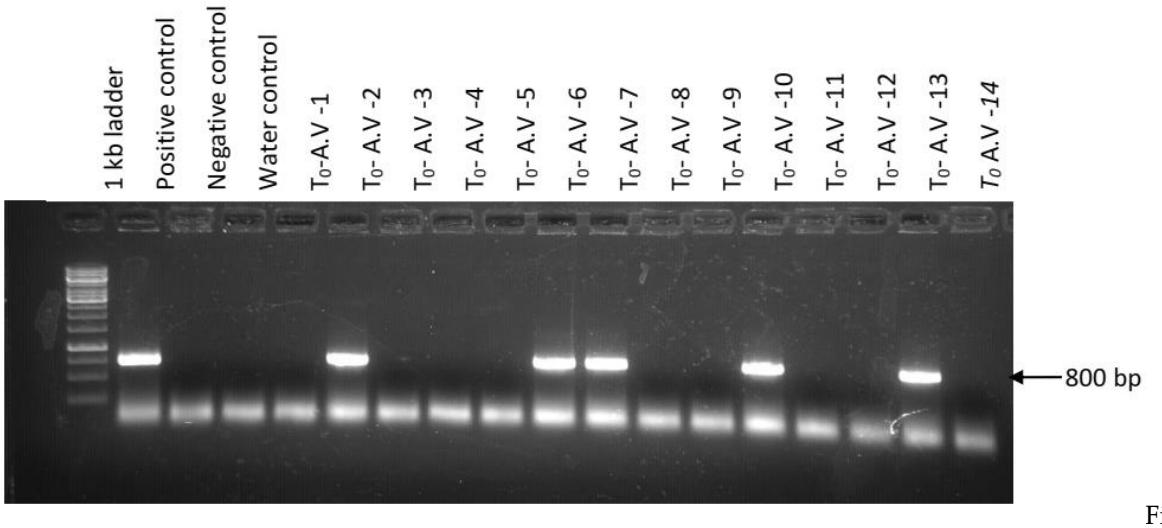


Fig 3b: PCR analysis of putative tomato *cv*. Arka Vikas transformants transformed with pC2300-*tp2AX1* for the presence of *cry2AX1* gene.

ELISA Analysis

PCR positive plants were further subjected for the quantification of the insecticidal Cry2AX1 protein by using quantitative ELISA Kit. Nine were found to be positive for the expression of *cry2AX1* and the concentration of Cry2AX1 protein ranged from 0.022 to 0.359 µg/g of fresh leaf tissue (Table 1) out of twenty-eight plants.

Table 1: Quantitative ELISA of T_0 transformants of tomato transformed with pC2300-*2AX1* construct

S. NO	T_0 transgenic plant	Concentration of Cry2AX1 protein (µg/g fresh leaf tissue) Mean ±SD	Mortality of *H. armigera* neonates (%) (Mean ± SD)
1	T_0-A.V-10	0.312 ± 0.01	89.30 ± 3.67
2	T_0-A.V-16	0.093 ± 0.00	63.77 ± 2.71
3	T_0-A.V-23	0.329 ± 0.01	90.01 ± 2.21
4	T_0- A.V -29	0.311 ± 0.03	88.39 ± 2.41
5	T_0- A.V -39	0.022 ± 0.01	16.67 ± 0.17
6	T_0- A.V-40	0.057 ± 0.01	36.67± 0.01
7	T_0- A.V -44	0.267 ± 0.03	67.67 ± 1.90
8	T_0-A.V-56	0.359 ± 0.03	90.00 ± 2.11
9	T_0- A.V -72	0.269 ± 0.03	66.67 ± 0.81
10	Control	0.0	0.0

Insect Bioassay

The mortality of neonates on T_0 plants ranged from 16.67 to 90 per cent, whereas the control plants showed no mortality (Table 1). There was growth inhibition in surviving larvae and a high reduction in leaf area feeding was observed in the ELISA positive plants whereas larvae released on control plants were alive with normal growth (Figure 4)

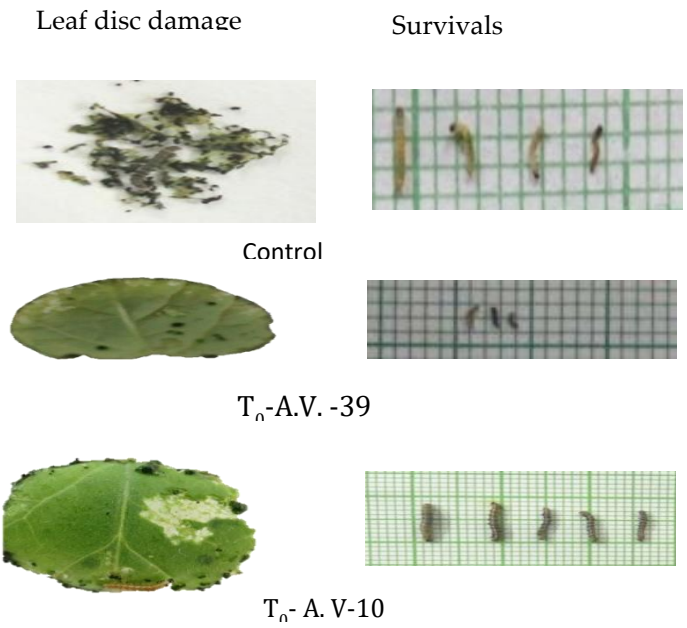


Figure 4: Detached leaf bit bioassay against *H. armigera* in transgenic tomato plants expressing Cry2AX1 protein.

4.0. Discussion

Tomato, which is regarded as a genetic model for improving dicotyledonous plants is still regarded as a recalcitrant crop for transformation [16]. The average transformation frequency recorded ranged from 6.2 - 10.4% depending on regeneration protocol, transformation procedure and genotype [17]. In this study, a total of seventy six putative transgenic plants were regenerated with a regeneration efficiency of 15.17% and a transformation efficiency of 36.84 %. Most published protocols for tomato transformation [18, 19, 20, 21] had described co-cultivation of the explants with various *Agrobacterium* strains (LBA4404, C58C1, GV311SE or A208) for 48hrs with bacterial densities ranging from 10^8 to 5.0x 10^8 cells/ml gave variable transformation efficiencies ranging from 3.5 to 28 % with different cultivars of tomato. The regeneration frequency obtained in this study is low when compared to the results of other earlier workers like Rajesh *et al.* [22] observed transformation efficiency of 58 per cent when using the medium supplemented with zeatin 2 mg/l and IAA 0.1 mg/l in tomato cultivar Arka Vikas. This disparity in transformation efficiency of tomato is subject to many factors such as the cultivar, type of explants and its age, *Agrobacterium* strain and its density, co-cultivation time and regeneration medium [3, 23]. Also, frequency of transformation may change with the actual exercise.

Twenty-eight were confirmed positive for the presence of the gene of interest out of the seventy-six regenerated plants and subsequently only eight were found to contain Cry2AX1 protein, ranging from 0.022 to 0.359 µg/g of fresh leaf tissue. A wide range of Bt protein expression in transgenic plants were reported by several earlier workers and independent plants of same genetic background and gene construct show greater differences in the level of expression. The fluctuating level of Cry2AX1 protein detected among different transgenic lines could be envisioned for the relative location of the integrated T-DNA into the genome, called position effects, also the chromatin structure; methylation state; post-transcriptional regulation and copy numbers [24,]. Furthermore, truncations and rearrangements have also been reported to impact the expression of the transgene [7]. The study also recorded many transgenic lines which were positive by PCR but did not have expression of Cry2AX1 protein at all this could be due to the total inactivation of the gene because of its integration into highly repetitive DNA region of the plant's genome. Integration site of transgene in the genome may have a negative effect on its expression. Significant differences in the level of expression of the *Bt* protein among the transgenic T_0 plants have been reported earlier [16, 22, 25].

A positive correlation was observed between the level of Cry2AX1 expression and mortality and reduction in feeding against *H. armigera* larvae. Such positive correlations between expression level of Cry protein and insect mortality have been reported in many studies [26, 27]. In the present study, the level of expression of Cry protein also showed a positive relationship with insect mortality. Differences in the level of mortality observed among the different transgenic lines could be ascribed to variation in the level of *Bt* gene expression. The surviving larvae on transgenic lines showed severe growth inhibition and significant differences were observed in the mortality percentage of neonate larvae between the transgenic and non-transformed tomato leaf bits.

5.0. Conclusion

A chimeric Bt gene, *cry2AX1*, was mobilized into tomato through *Agrobacterium* – mediated transformation method and tomato plants expressing the gene were generated. Significant mortality of *H. armigera* neonates was observed when exposed to the developed transgenic tomato leaves. This establishes that Cry2AX1 could be one of the most effective *Bt* genes against lepidopteran insect pests of tomatoes.

Author Contributions: "Conceptualization, BEI. and SD.; methodology, BEI and SD.; software, BOM; validation, BEI. and SD and BOM.; formal analysis, BEI..; investigation, BEI.; resources, SD.; data curation, BI.; writing—original draft preparation, BEI and BI.; writing—review and editing, SD.; visualization, BI.; supervision, SD.; project administration, BOM.; funding acquisition, BEI.

References

1. Sandhya, D.; Jogam, P.; Venkatapuram, A. K.; Savitikadi, P.; Peddaboina, V.; Allini, V. R.; Abbagani, S. Highly efficient Agrobacterium-mediated transformation and plant regeneration system for genome engineering in tomato. *Saudi Journal of Biological Sciences* **2022**, *29*(6),
2. Costa, J. M.; Heuvelink, E. P. The global tomato industry. In *Tomatoes* **2018** *27*, pp. 1-26). Boston, USA: CABI.
3. VanEck, J; Keen, P; Tjahjadi, M. *Agrobacterium* tumefaciens-Mediated Transformation of Tomato. *Methods Mol Biol*. **2019**, *1864*:225-234.
4. Agrawal, S.; Rami E. A Review: Agrobacterium -mediated gene transformation to increase plant productivity. *J Phytopharmacol* **2022**; *11 (2)*:111 -117. doi: 10.31254/phyto.202 2.1 1211
5. Danial, G. H.; Ibrahim, D. A.; Song, G. Q. *Agrobacterium*-mediated transformation of two tomato cultivars (*Lycopersicon esculentum mill*.) cv. Sandra and Rocky. *Iraqi Journal of Agricultural Sci.*, **2018** *52*(3), 745-755**.**
6. Kumar, P.; Kamle, M.; Borah, R.; Mahato, D. K.; Sharma, B. *Bacillus thuringiensis* as microbial biopesticide: uses and application for sustainable agriculture. *Egyptian Journal of Biological Pest Control*. **2021** *31*(1), 1-7.
7. Sun, S.; Kang, X. P.; Xing, X. J.; Xu, X.Y.; Cheng, J.; Zheng, S.W.; Xing. G.M. *Agrobacterium*-mediated transformation of tomato (*Lycopersicon esculentum* L. cv. Hezuo 908) with improved efficiency. Biotechnology & Biotechnological Equipment. **2015** *3;29(5)*:861-8.
8. Nderitu, W.P.; John, J.M.; Otieno, M.; Arunga, E.; Mattias, J. Tomato leaf miner (Tuta absoluta)(Meyrick 1917)(Lepidoptera: Gelechiidae) prevalence and farmer management practices in kirinyanga county, Kenya. *Jour. of Ento and Nematolo*. **2018** *10(6)*:43-9.
9. Bamishaiye, E.I.; Varanavasiappan, S.; Balakrishnan, N.; Udayasuriyan, V.; Sudhakar, D. Genetic Manipulation of Tomato (*Solanum lycopersicum*) cv. PKM-1 using *cry2AX1* Gene for Insect Resistance. *Int.J.Curr.Microbiol.App.Sci* **2017***.6(12),* 4309-4319.
10. Sandhya, D.; Jogam, P.; Venkatapuram, A. K.; Savitikadi, P.; Peddaboina, V.; Allini, V. R.; Abbagani, S. Highly efficient *Agrobacterium*-mediated transformation and plant regeneration system for genome engineering in tomato. *Saudi Jour of Bio Sci*. **2022** *1;29(6)*,103292.
11. Sushmitha, J.; Bamishaiye, E.I.; Balakrishnan, N.; Varanavasiappan, S.; Arul, L.; Kumar, S. M.; Joel, A.J.; Sudhakar, D. Efficacy of cry2AX1 gene in transgenic tomato plants against Helicoverpa armigera and Spodoptera litura. *Madras Agric J.* **2018** 16;105:341-5.
12. Hanur VS, Reddy B, Arya VV, Rami Reddy PV. Genetic transformation of tomato using Bt Cry2A gene and characterization in Indian cultivar Arka Vikas. *J. Agr. Sci. Tech.* **2015** *17*: 1805-1814
13. Udayasuriyan, V.; Arulselvi, P.; Balasubramani V.; Sudha, D.; Balasubramanian, P.; Sangeetha, P.. Construction of new chimeric cry2AX1 gene of *Bacillus thuringiensis* encoding protein with enhanced insecticidal activity. *Indian patent***. 2010** 244427
14. Ruturaj, R.B.; Kumar, A.N.; Nandeesha, P.; Manikandan, R.; Balakrishnan, N.; Balasubramani, V.; Sudhakar D.; Udayasuriyan. V. Transformation of tomato with *cry2AX1* gene of *Bacillus thuringiensis*. Int. J. Tropical Agric., **2014.** 32**:** 3-4.
15. Doyle, J.J.; Doyle, J.L. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin*, **1987** *19*, 11-15.
16. Prihatna, C.; Chen, R.; Barbetti, M.J.; Barker, S. J. Optimisation of regeneration parameters improves transformation efficiency of recalcitrant tomato. *Plant Cell, Tissue and Organ Culture (PCTOC).* **2019** 137(3):473-83.
17. Nonaka, S.; Someya, T.; Kadota, Y.; Nakamura, K.; Ezura, H. Super-*Agrobacterium* ver. 4: Improving the transformation frequencies and genetic engineering possibilities for crop plants. *Frontiers in plant science*, **2019** *10*, 1204.
18. McCormick S, Niedermeyer J, Fry J, Barnason A, Horsch R, Fraley R. Leaf disc transformation of cultivated tomato (*L. esculentum*) using *Agrobacterium tumefaciens*. *Plant cell reports.* **1986** *5(2)*:81-4.
19. Hamza, S.; Chupeau, Y.. Re-evaluation of conditions for plant regeneration and *Agrobacterium* mediated transformation from tomato (*Lycopersicon esculentum*). *J. Exp. Bot.*, **1993** *44***:** 1837-1845.
20. Vidya, C.S.S.; Manoharan, M.; Kumar, C.T.R.; Savithri H.S.; Sita. G.L.. *Agrobacterium-* mediated transformation of tomato (*Lycopersicon esculentum* Mill. var.Pusa Ruby) with coat protein gene of physalis mottle tymovirus. *J. Plant Physiol.*, **2000** *156***:** 106-110.

21. Sharma, M.K.; Solanke, A.U.; Jani, D.; Singh, Y.;. Sharma, A.K. A simple and efficient *Agrobacterium*-mediated procedure for transformation of tomato. *J. Biosci.,* **2009** *34(3):* 423-433.

22. Rajesh, N.; J. Sivakumar, P.J.E. Prasanth and P.O. Basha. 2016. An establishment of efficient *Agrobacterium* mediated transformation in tomato (*Solanum lycopercicum*). **Int. J. Recent Sci. Res., 7(1):** 8583-8591.

23. Gougerdchi, V.; Dorani, E.; Valizadeh, M.; Zamani, M. Optimization of gene transfer with *Agrobacterium* to Bonab tomato cultivar using chitinase gene. *Genetic Engineering and Biosafety Journal*. **2022** *11(1)* 16-25.
24. Ingham, D.J.; Beer, S.; Money, S.; Hansen, G. Quantitative real-time PCR assay for determining transgene copy number in transformed plants. *Biotechniques*. **2001** *31(1)*:132-40.
25. Dalla, C. L.; Vinciguerra, D.; Giacomelli, L.;, Salvagnin, U.; Piazza, S.; Spinella, K.; Malnoy, M.; Moser, C.; Marchesi, U. Integrated approach for the molecular characterization of edited plants obtained via *Agrobacterium tumefaciens*-mediated gene transfer. Eur Food Res and Technol. **2022** *248*(1):289-99.
26. Amiri, A. N.; Bakhsh, A. An effective pest management approach in potato to combat insect pests and herbicide. *3 Biotech*. **2019** *9(1)*:1-2.
27. Jamil, S.; Shahzad, R.; Rahman, S.U.; Iqbal, M. Z.; Yaseen, M.; Ahmad, S.; Fatima, R. The level of Cry1Ac endotoxin and its efficacy against *H. armigera* in Bt cotton at large scale in Pakistan. *GM crops & food*. **2021** *2;12(1)*:1-7.