Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2019; 7(3): 1314-1321

Received: 29-03-2019
Accepted: 30-04-2019

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Cloning and characterization of insecticidal *cry/vip* genes from an indigenous *Bacillus thuringiensis* isolate T29 and evaluation of its toxicity to maize fall armyworm *Spodoptera frugiperda*

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Abstract
Bacillus thuringiensis (*Bt*), Gram-positive, spore forming bacterium produces insecticidal proteins during vegetative phase or sporulation phase which are toxic to a wide range of insects and are encoded by *cry* and *vip* genes. Identification of genes encoding insecticidal protein is important and will be useful in generating insect resistance transgenic plants. In this present study, an indigenous *Bt* isolate T29 was characterized and the lepidopteran toxic proteins encoding genes were cloned. The colony of *Bt* isolate (T29) was creamy white in colour with fried egg type and circular in shape with serrate margin and the isolate had bipyramidal and cuboidal shaped parasporal inclusions. Bioassay studies with spore-crystal mixture of T29 *Bt* isolate showed 100 per cent mortality against the invasive pest Fall armyworm *Spodoptera frugiperda*. SDS-PAGE analysis revealed the presence of two insecticidal crystal proteins with molecular weight of ~130 kDa (Cry1) and ~65 kDa (Cry2). The isolate showed the presence of *cry1*, *cry2Aa* and *vip3A* genes while screening by polymerase chain reaction (PCR) using gene specific primers. The lepidopteran toxic genes *viz*., *cry1Ac* (truncated), *cry2Aa* (full length) and *vip3A* (partial) genes were cloned and sequenced.

Keywords: *Bacillus thuringiensis*, characterization, *cry* and *vip* genes, bioassay, *S. frugiperda*

Introduction
Bacillus thuringiensis (*Bt*) is an entomopathogenic Gram-positive spore forming bacterium which secretes insecticidal proteins during sporulation and vegetative stages. These are highly toxic to various insect pests but not harmful to other vertebrates and human beings and it is used as biological insecticide [1]. Intensive use of chemical insecticides has caused major problems including outbreak of insect pests and public health and environmental concerns in several countries. So, microbial insecticides can act as an alternative to chemical insecticides. Initially these insecticides were applied on plants as liquid spray but the major disadvantage was its rapid inactivation by environmental factors. Transgenic plants containing these *Bt* protein(s)/Cry protein(s) is an alternative for these demerits [2].

The ability of *Bt* is to produce two major groups of insecticidal proteins pertaining to Cry and Vip toxins. Cry proteins are produced during the sporulation phase and accumulated as parasporal crystalline inclusions and vegetative insecticidal proteins (Vip) are produced during the vegetative phase of growth and are secreted into the culture medium [3]. The proteins were classified based on the homology of their amino acid sequence [4]. Till date 78 *cry* genes, 4 *vip* genes and 3 *cyt* gene family has been identified (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/ toxins2.html). The crystal proteins *viz.*, Cry1, Cry2 and Cry9 proteins [3, 5] and Vip3A [6] were found to be toxic to larvae of various lepidopteran insect pests. Most of the Cry proteins are protoxins in nature that bind to receptors present in the susceptible larval midgut epithelial cells and get activated after proteolytic cleavage which leads to cell disruption and death of the larvae [7]. They are highly specific towards lepidopteran, coleopterans, dipterans and also some of the nematodes [8]. Though Cry proteins and Vip proteins follow the same mode of action or sequence of events in causing the mortality, they are structurally dissimilar and have different binding sites. Combination of these toxins with diverse mode of action is considered as an efficient strategy to broaden the insecticidal

spectrum and delay the development of insect resistance [9]. It is also being considered in the development of insect resistant transgenic plants through gene pyramiding strategy [10].

Feitelson *et al.* [11] reported that the molecular effectiveness of Bt toxins is 300 and 80000 times higher than synthetic pyrethroids and organophosphates, respectively. The genetic engineering technologies helped in transforming the plants with insect resistant genes and enabled the plants to show in-built resistance against target insect pests. To counteract the resistance in plants, insects adapted some mechanisms and became resistant to the transgenic plants. There have been reports of field evolved resistance against *Bt* proteins in many countries. For example, the pink bollworm *Pectinophora gossypiella* Saunders (Gelechiidae, Lepidoptera) in *Bt* cotton has been reported recently to have evolved resistance to two *Bt* proteins Cry1Ac and Cry2Ab [12]. Likewise, it has been reported that maize Fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Noctuidae, Lepidoptera) in corn has evolved resistance towards Cry1F protein [13]. Molecular characterization of *Bt* (i.e.) studies on parasporal inclusions, detection of *cry* and *vip* gene content, cloning the genes, expression of genes in bacterial systems and studying the toxicity will be helpful in identifying gene(s) responsible for developing insect resistant transgenic plants. Hence characterization of *Bt* isolate(s), identification of novel gene(s) and studying the insecticidal property should be attempted regularly for deploying the novel gene(s) in development of insect resistant transgenic plants where insects have developed resistance against the existing *Bt* proteins. In this present study, an indigenous *Bt* isolate T29 was characterized, lepidopteran toxic *cry* and *vip* genes were identified, cloned, sequenced and a preliminary bioassay was conducted against the invasive pest to India, *S. frugiperda*.

2. Materials and Methods

2.1 Bacterial strains and plasmids

An indigenous *Bt* isolate T29, reference strain *Bt* subsp *kurstaki* (HD1) and acrystalliferous *Bt* strain (4Q7) were revived from the glycerol stock being maintained at *Bt* lab in the Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore, India. The reference strain HD1 and acrystalliferous *Bt* strain 4Q7 were originally obtained from Bacillus Genetic Stock Centre, Ohio University, Columbus, Ohio, USA. *E. coli* DH5α was used for maintaining plasmid construct. The T/A cloning vector, pGEM-T Easy vector was purchased from (Promega vector system I, USA) was used in this study.

2.2 Colony, crystal morphology and SDS-PAGE analysis

Colony morphology of the *Bt* isolate T29 was examined by visual observation. The spore-crystal mixture from T29 *Bt* isolate was harvested according to the procedure followed by [14]. A loopful of *Bt* isolate T29 was inoculated into 5 ml of T_3 broth and incubated at 30 °C for overnight. After 12-14 hours incubation, 1 per cent of cell culture was transferred to 25 ml of T_3 broth and incubated at 30 °C and 180 rpm about 48 - 60 hours. After incubation a drop of inoculum was taken and smeared on glass slide and heat fixed, stained and crystalline inclusion was observed under bright field microscope (Leica DM 1000LED, DFC295, Germany) with 100X magnification for observing the shape of the crystals.

The protein was harvested once the cell crossed 90 per cent sporulation. The 90% sporulated culture was transferred to 4 °C at least half an hour before harvesting, centrifuged at 4 °C and the pellet was resuspended in 25 ml of ice-cold Tris–EDTA buffer and washed thrice with Tris-EDTA buffer containing PMSF (phenyl methyl sulphonyl fluoride) and once with NaCl solution (0.5mM) [Tris 10 mM, EDTA 1 mM, pH 8.0 with 1 mM PMSF]. Finally, spore- crystal pellet was suspended in 100 µl of sterile distilled water containing 1 mM PMSF and stored at -20 °C [14] Then the protein profiling was done by SDS-PAGE analysis according to the standard protocol [15] using 10% running and 4% stacking gels. The gels were stained with 0.4% Coomassie Blue R250. The molecular mass of proteins was determined using a higher range protein molecular weight marker (GeNeiTM, Bengaluru, India).

2.3 Bioassay

The insect, *S. frugiperda* culture was maintained in natural host in *Bt* laboratory, Department of Plant Biotechnology, CPMB&B, TNAU, Coimbatore. The insecticidal activity of proteins containing *Bt* isolate T29 was assessed by performing the bioassay against *S. frugiperda* using spore-crystal mixture by leaf disc surface coating method. The spore-crystal mixture of *Bt* isolate T29 was coated on leaf disc. Young maize leaves were freshly collected from the field, thoroughly washed with distilled water and allowed to dry. The leaf disc was cut into 2x2 cm size and 20µl of crude spore crystal mixture was coated on surface of leaf and allowed to dry. The newly emerged larvae were starved for 2 hours and ten neonate larvae were released on the leaf disc using camel hair brush without any physical injury. The leaf disc was placed in a Petriplate with a diameter of 9 cm. One larva was released per leaf disc. Thirty larvae were used for each isolate. A control was also maintained for the study. The observations on mortality (%) were recorded after 48 hours of the treatment with an interval of 24 hours up to seven days.

2.4 PCR screening of *cry1, cry2Aa* and *vip3A* genes

Genomic DNA was isolated from the indigenous *Bt* isolate T29 and reference strain HD1[16] and for Polymerase Chain Reaction (PCR) in thermal cycler (Eppendrof Mastercycler Personal, Germany). A total of 25µl reaction volume contains 1ng of genomic DNA of *Bt,* 1 µM of forward and reverse primers, 2.5mM of dNTPs, 2.5µl of 10X *Taq* buffer and 1.5 units of *Taq* DNA polymerase enzyme. The gene specific primers (5' to 3') CATGATTCATGCGGCAGATAAAC (forward) and TTGTGACACTTCTGCTTCCCATT (reverse) for *cry1* [17], GTTATTCTTAATGCAGATGAATGGG (forward) and GAGATTAGTCGCCCCTATGAG (reverse) for *cry2Aa* [17] and ACATCCTCCCTACACTTTCTAATAC (forward) TCTTCTATGGACCCGTTCTCTAC (reverse) for *vip3A* [18] were used for the detection. The PCR was performed for 30 cycles and PCR profile follows 94 °C for 2 minutes, 94 °C for 1 minute, 62 °C, 60 °C and 50 °C for 45 seconds (for *cry1, cry2Aa* and *vip3A* respectively), 72 °C for 1 minute and 72 °C for 7 minutes.

2.5 Amplification of truncated *cry1Ac* and full length *cry2Aa*

Amplification of truncated *cry1Ac* and full length *cry2Aa* genes were performed by PCR using HF polymerase. The primers (5'-3') GCCCCGGGCCTGGGTCAAAAATTGATATT TAG (forward) and (CGGGTCGACTAAATTGGATACTTGATCA) (reverse) for

amplification of truncated *cry1Ac* (~2.1 kb) [19] and ATGGTACCATGAATAATGTA TTGAATAGTGGAA (forward) and GTTCTAGACTCAAACCTTAATAAAGTGGTG (reverse) for amplification of full length of *cry2Aa* (~1.9 kb) [20] were used and (1.9 kb) with size of ~2.1 kb and 1.9 kb respectively. The amplified PCR products were column purified with PCR clean up kit (Thermo Scientific, USA) as per the instruction given in the user's manual.

2.6 Cloning of truncated *cry1Ac*, full length *cry2Aa* and *vip3A* partial genes from *Bt* isolate T29

The purified PCR products (truncated *cry1Ac*, full length *cry2Aa* and partial gene of *vip3A*) were 'A' tailed, ligated into pGEM-T Easy vector as per the manufacture's instruction. The ligated products were transferred into *E.coli* DH5α as per the standard protocol [21]. Transformants were screened by alpha complementation test and confirmed by colony PCR with gene specific primers for the presence of insert. Then the recombinant plasmid was isolated and sequenced.

2.7 Nucleotide sequencing of recombinant plasmids

The recombinant plasmids were isolated from the *E. coli* transformants harbouring *cry1Ac, cry2Aa* and *vip3A* genes of *Bt* isolate T29 and nucleotide sequencing of recombinant plasmids were done by automated sequencer (ABI 3730xl Genetics) at Eurofins Genomics India Pvt. Ltd. The forward and reverse sequences were edited by BioEdit software version 7.0.5 and sequences were analyzed using BLASTN. Based on the E- value and query coverage five hits were selected and used for comparing with the test isolates taken for this present study. Multiple sequence alignment was done by using Clustal Omega and Geneious softwares. Nucleotide sequences of truncated *cry1Ac*, full length *cry2Aa* and partial *vip3A* genes of *Bt* isolate T29 were deposited in GenBank.

3. Results

3.1 Colony and crystal morphology of indigenous *Bt* isolate

The purified single colony obtained from the *Bt* isolate T29 was fried egg type, creamy white in colour, circular shaped with serrate margin. After sub-culturing from single colony, a drop of inoculum (during sporulation stage) was taken in a glass slide, heat fixed, stained and observed under bright field microscope with 100X magnification. It revealed that the *Bt* isolate contained bipyramidal and cuboidal crystals (Fig. 1).

3.2 Bioassay against *Spodoptera frugiperda*

A prelimenary bioassay was performed to find out the insecticidal activity of *Bt* isolate T29 along with HD1 as positive control. The spore crystal-mixture was coated on the surface of the maize leaf disc and the first instar larvae were relased. Hundred per cent mortalilty was observed on both HD1 and T29 isolate under laboratory conditions (Fig. 2).

3.3 SDS-PAGE analysis for insecticidal protein

SDS –PAGE analysis of spore-crystal mixture isolated from the indigenous *Bt* isolate T29 showed the protein bands ~130 kDa and~ 65 kDa corresponding to Cry1 and Cry2 proteins like HD1 reference strain (Fig. 3).

3.4 PCR screening of lepidopteran toxic genes *cry1, cry2Aa* and *vip3A*

Screening by PCR for the presence of the lepidopteran toxic genes *viz., cry1, cry2A* and *vip3A* showed amplification at~ 290 bp, ~490 bp and ~680 bp, respectively for the Bt isolate T29 and reference strain (HD1) (Fig. 4).

3.5 Full length and truncated gene amplification by PCR

Truncated *cry1Ac* and full length *cry2Aa* and genes showed amplifications of ~1.9 kb and ~2.1 kb, respectively (Fig. 5).

3.6 Cloning and colony PCR for confirmation of transformants

The PCR products of truncated *cry1Ac* (~2.1 kb), full length *cry2Aa* (~1.9 kb) and partial *vip3A* genes were individually ligated to pGEM®- T easy vector and transferred into *E.coli* DH5α. The positive clones were confirmed by colony PCR with gene specific primers (Fig. 6). The result of colony PCR indicated that the corresponding insert(s) are present in the clone(s). Positive clones were selected and recombiant plasmids were isolated and sequenced.

3.7 Nucleotide sequencing

The nucleotide sequence analysis showed 98 to 99 per cent homology to *cry1Ac* and this 3' truncated *cry1Ac* gene (2105 bp) from *Bt* isolate (T29) revealed the presence of upstream 147 bp and internal region (1958 bp) corresponding to *cry1Ac* gene starting from ATG which encoded 652 amino acids. The top five hits were selected (GenBank ID: AAB49768.1, AAN07788.1, ABB89046.1, ALT07695.1 and ACV81774.1) while doing BLASTN analysis based on the E-value and query coverage and compared with the sequence of truncated *cry1Ac* gene obtained for T29 *Bt* isolate. The results of multiple sequence alignment revealed that the deduced amino acids sequence of truncated *cry1Ac* of T29 *Bt* isolate, differed at three positions (i.e.) at 453rd position phenylalanine to leucine, at 517th position proline to alanine and at 626th position asparagine to therionine were found in *Bt* isolate T29.

The nucleotide sequence analysis of full length *cry2Aa* gene (1902 bp) from *Bt* isolate T29, starting from ATG encoded 634 amino acids. The nucleotide analysis showed 98 to 99 per cent homology with the already available sequences in NCBI and the top most hits (GenBank ID: AYN72229.1, AYN72230.1, AYN72231.1, AYN72232.1 and AYN72233.1) were selected based on the E-value and query coverage and compared. Multiple sequence alignment was carried out using Clustal Omega, deduced amino acids sequence of *cry2Aa* differing at five positions (i.e.) at 19th position valine to leucine, at 233th glycine to glutamic acid, at 299th leucine to phenylalaine, at 340th position valine to aspartic acid 414th position asparagine to aspartic acid (Fig. 7A). However, present study amino acid sequence of truncated *cry1Ac*, differed at seven positions with GenBank ID: ACV81774.1 at 45th position histidine to glutamine, at 61th position glycine to valine, at 62th position glutamic acid to aspartic acid, at 277th position glycine to aspartic acid, at 293th position threonine to serine, at 453th position phenylalanine to leucine and at 517th position proline to alanine.

The nucleotide sequence analysis of partial *vip3A* gene (675 bp) obtained from T29 *Bt* isolate showed 98 to 99 per cent homology with the already available sequences in NCBI. The top most *vip3A* hits (GenBank ID: AGL75708.1, AGL75706.1, AGL75707.1, AGL75709.1 and AGL75712.1) were selected based on the E-value and query coverage and compared. Multiple sequence alignment was carried out using Clustal Omega software. The nucleotide sequence of *vip3A* of T29 isolate differed at 108th position (Thymine to Cytosine).

The nucleotide sequence of truncated *cry1Ac*,full length *cry2Aa* and partial gene of *vip3A* of *Bt* isolate T29 were submitted in NCBI and GenBank Accession numbers are MK882923, MK813910 and MK530688, respectively.

4. Discussion

Bt is a naturally occurring Gram-positive bacteria have been used for insect control [22]. It is reported that *Bt* were isolated from different habitats (i.e.) soil, leaf and insect cadaver [23] goat gut [24] and milk and mossy pine cone [25]. *Bt* producing insecticidal proteins were highly specific to lepidopteran, coleopteran, hymenopterans and nematodes [26]. *Bt* based biopesticides are alternative to chemical pesticides and it accounts about 80 per cent of all biopesticides marketed worldwide [27]. Characterization of *Bt* strains may lead to get novel proteins with higher insecticidal activity or broaden the insect spectrum.

In this present study, an indigenous *Bt* isolate T29 was characterized. It produced bipyramidal and cuboidal crystalline inclusions and proteins of ~130 kDa and ~65 kDa. In earlier reports also, most of the *Bt* isolates showed the bipyramidal and cuboidal shape of crystalline inclusions with same molecular weight proteins [20, 28, 29, 30, 31]. Arrieta *et al.* [32] reported a *Bt* strain CIBCM525 (isolated from coffee berry borer infested plantations) with bipyramidal and cuboidal shaped crystals and protein bands of ~130 kDa and ~65 kDa. Attahom *et al.* [33] reported that bipyramidal shaped crystal proteins have the specificity towards lepidopteran larvae. Bernard *et al.* [34] & Swamy *et al.* [29] found that bipyramidal form of crystals was more predominant and were more toxic to lepidopteran larvae. Yamamoto and Mclaughlin [35] reported that cuboidal crystals were toxic to lepidopteran and dipteran larvae.

Bt strain isolated form *Balaninus nucum* showed the presence of *cry1* and *cry2* genes and their corresponding proteins Cry1and Cry2 with ~130 kDa and ~65 kDa in SDS-PAGE analysis [36]. Spore-crystal mixture harvested from the 80 strains of *Bt* isolated from the Sichuan basin showed the protein bands ranging from 40-130 kDa belonging to major Cry protein family in SDS-PAGE analysis [37]. Ramalakshmi and Udayasuriyan [14] identified 17 out of 70 *Bt* strains producing proteins with ~135 kDa and ~65 kDa. Reyaz *et al.* [30] reported that five *Bt* strains (SWK1, KS2-3, 2M-6, KS2-6 and QZ-19) from Kashmir valley were positive for lepidopteran active genes of *cry1, cry2Aa* and *cry2Ab* found to have the insecticidal proteins of ~135 kDa and~65 kDa.

Seifinejad *et al.* [38] conducted a preliminary bioassay against *H. armigera* and found that four YD5, KON4, BR4 and 90 showed more mortality and less LC_{50} value compared to HD1 reference strain. Sahin *et al.* [39] recorded the highest level of toxicity against *S. littoralis*, *S. exigua* and *O. nubilalis* in *Bt* isolates encoding *cry* and *vip* genes. Boonmee *et al.* [31] characterized 511 *Bt* isolates in Thailand, detected the presence of lepidopteran toxic genes using PCR and found that the molecular mass of the proteins from ~65 to ~130 kDa though SDS-PAGE analysis.

Worldwide, detection of specific insecticidal genes in *Bt* isolates is performed using polymerase chain reaction (PCR) for screening and characterization of effective *Bt* isolates [40]. In this present study, *Bt* isoalte T29 was positive for lepidopteran toxic genes *cry1, cry2Aa* and *vip3A* in PCR analysis. Several scientists identified the presence of the genes in *Bt* isolates through PCR screening [31, 38, 10, 41, 42].

After PCR screening and knowing the presence of the insectidal genes, full length *cry2Aa,* truncated *cry1Ac* and partial gene of *vip3A* genes were amplified and ligated into pGEM-T vector, cloned and recombinant plasmids of *cry2Aa, cry1Ac,* and *vip3A* were sequenced. In earlier reports, the *Bt* isolate carrying *cry1Ac* showed variation in minimum of 5 positions and maximum of 19 position variation in amino acid sequence [43]. Manikandan *et al.* [20] reported that single amino acid variation at 46th position in the C- terminal region of *cry2Aa.* Lee *et al.* [44] reported that Cry1Ac proteins differing at only two amino acids position results in a 10 fold difference in toxicity against gypsy moth, *Lymantria dispar.* Similarly in this present study, we also observed variation in amino acids sequence of *Bt* isolate T29 carrying Cry1Ac, Cry2Aa and Vip3A proteins. Schnepf *et al.* [22] reported that slight changes in amino acid sequence within a Cry protein class can dramatically impact insecticidal activity. Hence studying about the presence of gene, variation in deduced amino acid sequence and insecticidal activity are important for evaluating a *Bt* isolate.

And however our amino acids sequence of truncated *cry1Ac* differed at seven positions with GenBank ID: ACV81774.1 at 45th position histidine to glutamine, at 61th position glycine to valine, at 62th position glutamic acid to aspartic acid, at 277th position glycine to aspartic acid, at 293th position threonine to serine, at 453th position phenylalanine to leucine and at 517th position proline to alanine was observed.

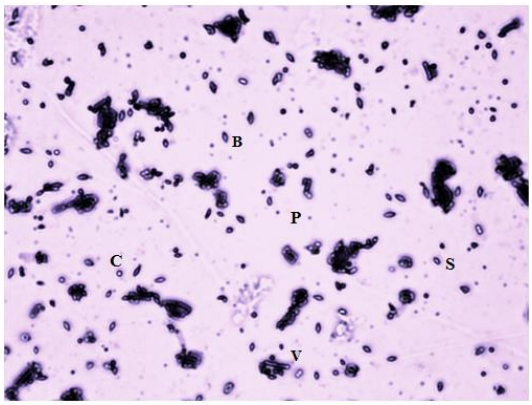
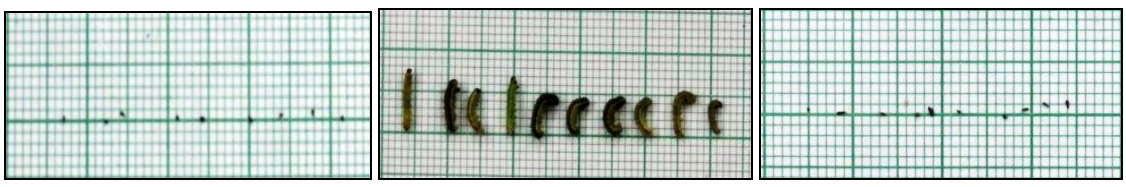


Fig 1: Crystalline inclusions of Bt isolate (T29) B = Bipyramidal crystals; C = Cuboidal; S = Spore; V = Vegetative cell; P = Protein



A. Control B. HD1 C. T29

Fig 2: Bioassay against *S. frugiperda* using spore-crystal mixture of the Bt isolate (T29)

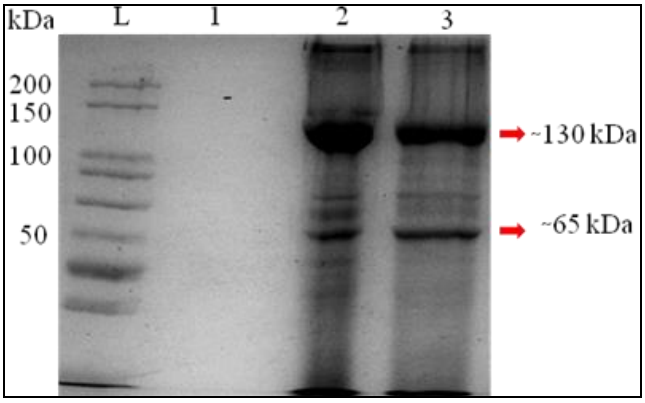
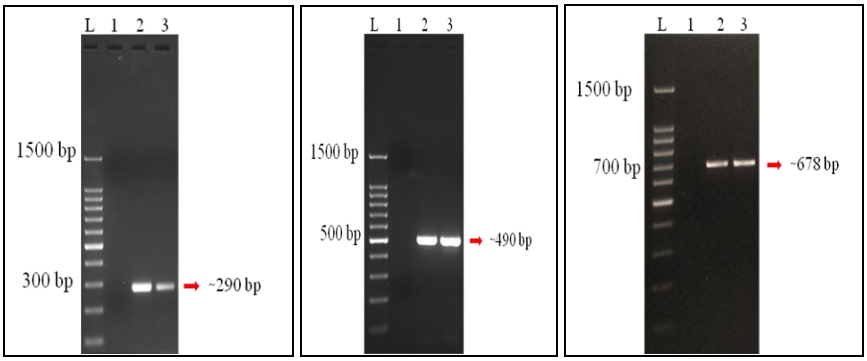
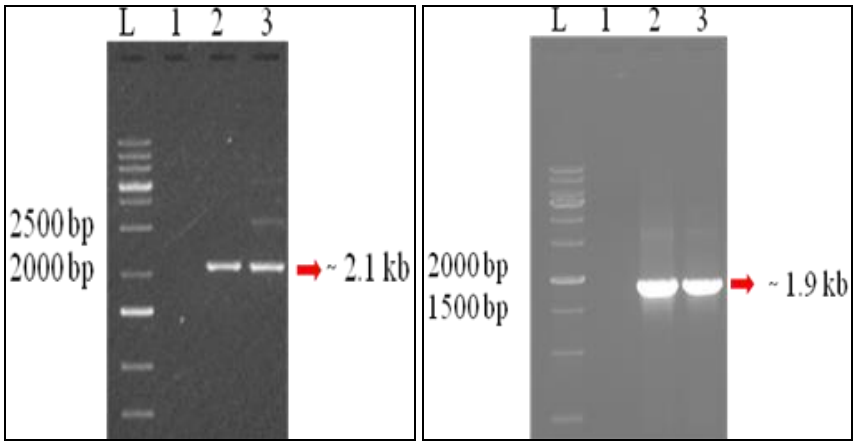


Fig 3: SDS-PAGE analysis of spore-crystal mixture of *Bt* isolate (T29) Lane L = Protein ladder; Lane 1 = Acrystalliferous 4Q7; Lane 2 = HD1; Lane 3 = T29



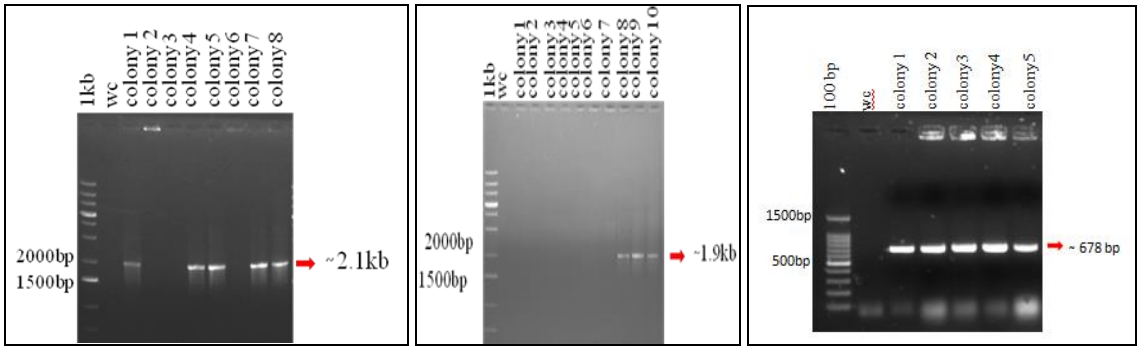
A. *cry1* B. *cry2Aa* C. *vip3A*

Fig 4: PCR screening for lepidopteran toxic genes Lane L = 100bp ladder; Lane 1. = Water contol; Lane 2. = HD1; Lane 3. = T29



A. Truncated *cry1Ac* gene B. Full length *cry2Aa* gene

Fig 5: Amplification of truncated/ full length *cry genes* in T29 Bt isolate



A. Truncated *cry1Ac* B. Full length *cry2Aa* C. Partial *vip3A*

Fig 6: Colony PCR for confirmation of transformants Lane L = 100bp ladder; Lane 1. = Water contol; Lane 2. = HD1; Lane 3. = T29

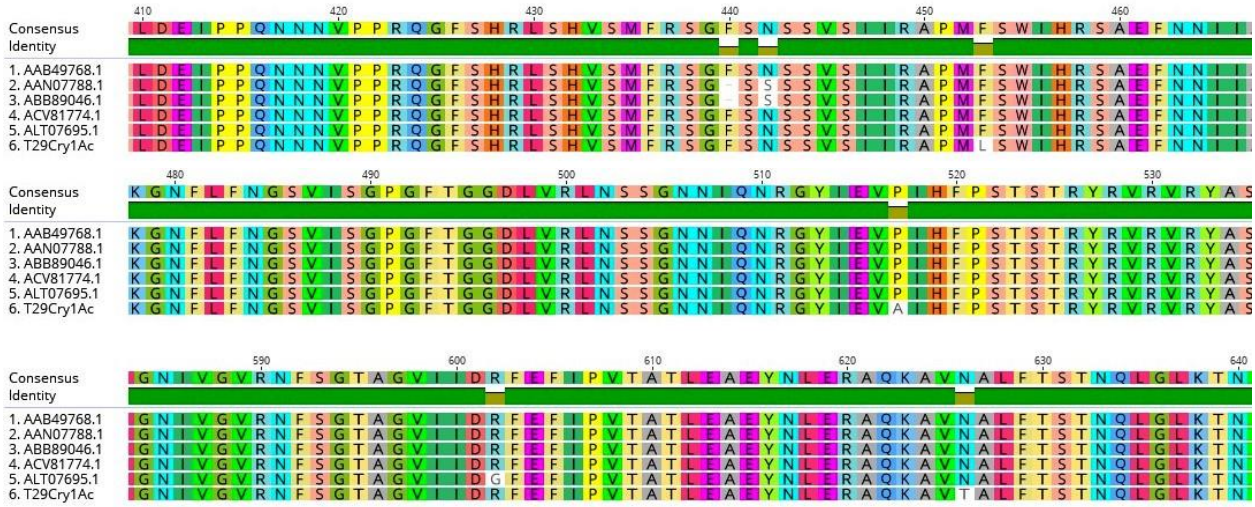


Fig 7A: Comparison of deduced amino acid sequence of *cry1Ac* gene of *Bt* isolate (T29) with other *cry1Ac* sequences

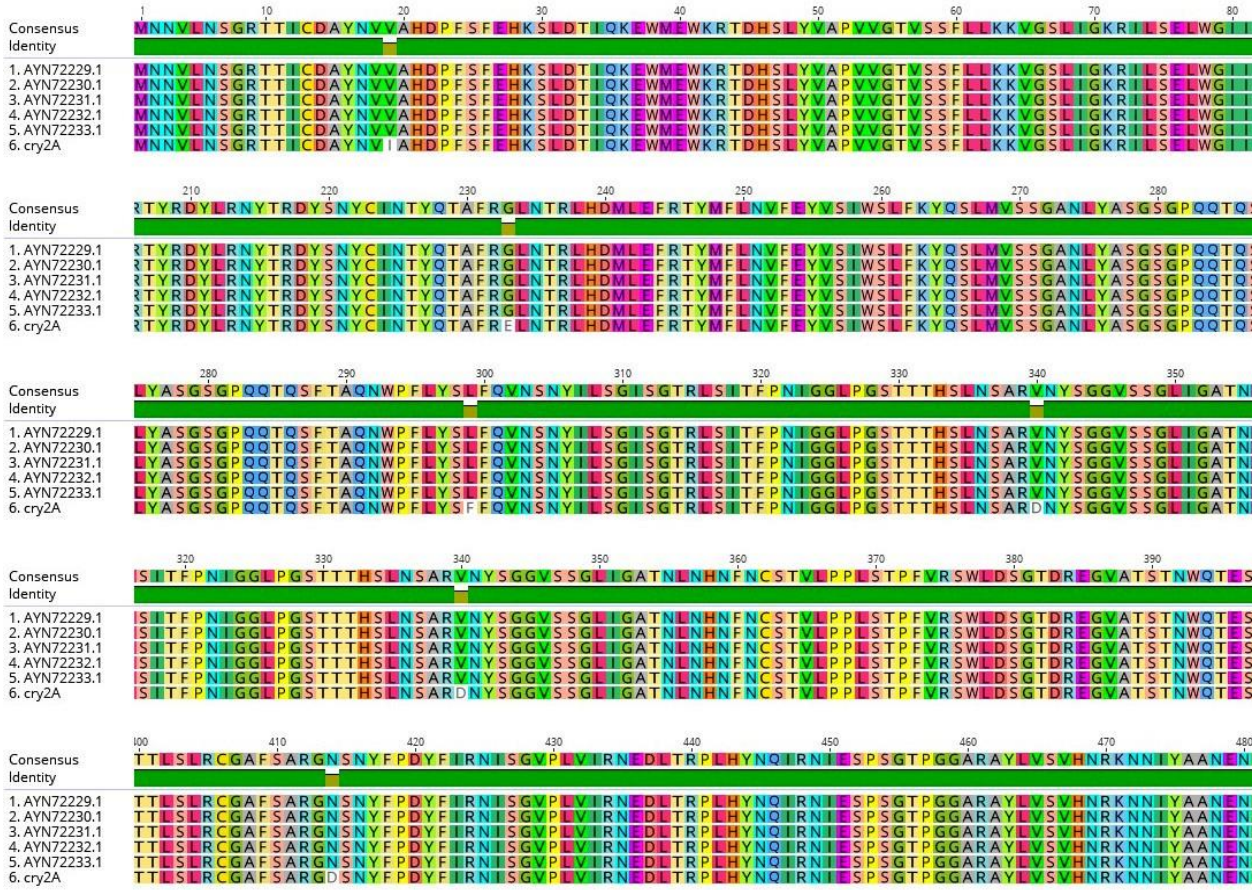


Fig 7B: Comparison of deduced amino acids sequences of *cry2Aa* gene of *Bt* isolate (T29) with other *cry2Aa* sequences

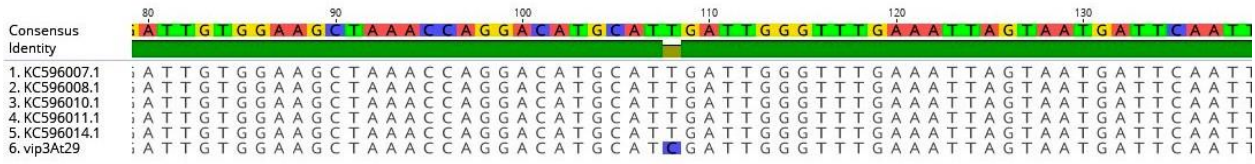


Fig 7C: Comparison of partial nucleotide sequences of *vip3A* gene of *Bt* isolate (T29) with other *vip3A* sequences

5. Conclusion

Characterization and screening for the presence of insecticidal genes in *Bt* isolate is important to find out a novel gene(s). In the presence study, it was found that indigenous *Bt* isolates T29 had *cry1Ac*, *cry2Aa*, *and vip3A* genes and the spore-crystal mixture of the isolate showed 100% mortality against the Fall armyworm, *S. frugiperda*. This isolate can be deployed for the management of *S. frugiperda*.

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