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# EVALUATION AND IDENTIFICATION OF NOVEL INSECTICIDAL TOXIN GENES FROM *BACILLUS THURINGIENSIS* (BERLINER) ISOLATES

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**ABSTRACT**

**The present study evaluated fifteen *Bacillus thuringiensis* (Berliner) isolates against second instar larvae of tobacco leaf eating caterpiller *Spodoptera litura* (F) and *Vip* genes were identified in the effective isolates. Four NCIM isolates (5111, 5112, 5116 and 5117) were found promising, and their $LC_{50}$ ranged from $2.5x10^5$ to $3.33x10^5$ ppm and $2.20x10^5$ to $2.85x10^5$ ppm in diet incorporation and surface contamination methods, respectively. The NCIM-5111 isolate exhibited the lowest $LC_{50}$ of $2.5x10^5$ ppm in diet incorporation and $2.2x10^5$ ppm in the surface contamination method. Surface contamination method revealed more mortality of 6.66 to 10.00% across the NCIM isolates. All the isolates were further used for identification of insecticidal toxin (*Vip, Cyt*) genes, and it was observed that four NCIM isolates harbour the *Vip3Aa1* gene and one viz., NCIM-5112 isolate carries the *Vip*1/ *Vip*2 gene, which has distinct motifs from the *Vip* toxins that are currently in use.**

**Key words:** Vegetative insecticidal protein, cytolytic protein, entomopathogenic bacterial isolates, *Spodoptera litura*, cellular extract, bioassay, *Vip* 1 and 2, surface contamination method, diet incorporation method, insecticidal proteins, $LC_{50}$

*Bacillus thuringiensis* (Berliner) an entomopathogenic gram-positive spore-forming bacterium. It produces delta-endotoxin proteins (*Cry* and *Cyt*) during sporulation with toxic activity against insects, nematodes and mites (Lopez et al., 2013; Jouzani et al., 2017). Certain strains of *B. thuringiensis* exude proteins into the growth medium during the vegetative development phase (Milne et al., 2008). The secreted insecticidal proteins divided two classes that were designated as vegetative insecticidal proteins (*Vip*) and (Crickmore et al., 2022) secreted insecticidal protein (Sip) (Donovan et al., 2006). These proteins exhibit strong insecticidal properties against numerous lepidopteran, coleopteran and some homopteran insects species (Sattar and Maiti, 2011). As a result, these are regarded as a fantastic additional source of *Cry* toxins for managing insecticide resistance (Gupta et al., 2021) and other benefits (Vazquez et al., 2021).

*Vip* proteins are categorized into four families based on the amino acid sequences. Some Coleoptera and Hemiptera pests are affected by *Vip*1 and *Vip*2 proteins, which function as binary toxins. The *Vip*2 component enters the cell and exhibits its ADP-ribosyl transferase activity against actin, inhibiting the formation of microfilaments, while the *Vip*1 component is thought to bind to receptors in the membrane of the insect midgut. *Vip*3 has no sequence similarity to *Vip*1 or *Vip*2 and is toxic to a variety of Lepidoptera and are especially toxic for species with low susceptibility to some *Cry*1A proteins (*Agrotis ipsilon* (*Hufnagel*), *Spodoptera frugiperda* (J E Smith) and *Spodoptera exigua* (*Hiibn*) (Chakroun et al., 2016). Several studies have shown that *Vip* proteins have a different mode of action when compared to insecticidal *Cry* proteins. *Vip* proteins have different receptor binding sites and ion channel properties and do not compete for binding sites with Cry genes (Gouffon et al., 2011; Syed et al., 2020). High insecticidal activity of *Vip* proteins have opened up the possibility of using them for pest control strategies to broaden the host spectrum activity of biopesticides based on *B. thuringiensis* toxins and thus facilitate the management of insect resistance to *B. thuringiensis* proteins and control of various insect pests (Mesrati

et al, 2011; Bravo et al., 2011). Therefore, research on finding novel *Vip* genes may provide alternatives and help alleviate insect resistance. Identification of suitable strains of bacteria and finding the novel toxins against a different group of insects is the need of hour to use in integrated pest management programs. In this context, the present study was undertaken to evaluate native and NCIM isolates of *B. thuringiensis* against the tobacco leaf eating caterpiller *Spodoptera litura* (F) using two methods of bioassay. Further, these isolates were screened for the presence of *Vip* and *Cyt* genes based on the polymerase chain reaction (PCR).

## MATERIALS AND METHODS

To determine the efficacy of *B. thuringiensis* isolates against *S. litura*, bioassay was performed using diet incorporation and surface contamination methods. Later, genes responsible for the insecticidal activity were identified by PCR *Spodoptera litura* was mass reared in the laboratory on castor leaves, adults were reared in insect rearing cages by providing water and honey as adult food. To prepare cell culture supernatants for bioassays, native *B. thuringiensis* isolates (BGC-1, BGC-2, RCM-2, GBP-2, GHB-1, MDH, KMS-2, GHM-1, GHM-2, MDS-2, HD-1) and National Collection of Industrial Microorganisms, NCL, Pune (NCIM) *B. thuringiensis* isolates (NCIM-5111, NCIM-5112, NCIM-5116, NCIM-5117) were grown on Luria agar medium at 37ºC for 48 hr. After incubation at 37ºC for 24-36 hr with shaking (250 rpm), the suspension was centrifuged at 10000 rpm for 10 min. Finally, the supernatant was transferred to a separate vial and kept at −20ºC (Sahin et al., 2018).

Cell culture supernatant of native and NCIM *B. thuringiensis* isolates were evaluated. The 100 µl of cell culture supernatant of bacterial suspension was mixed with 900 µl of semi-synthetic diet to makeup the concentration of $1x10^5$ ppm and the diet mixed with cell culture supernatant was poured into multi cavity trays, with approximately 4 ml/ well. *S. litura* second instar larvae were released into each well after being pre-starved for four hr. For each concentration, a total of 40 larvae were used at a replication rate of 10 larvae (4 replications). These trays were kept in the insectary at 25± 1°C, 70± 5.0 % RH, with a light: dark period of 16:8 hr. After treatment, observations on mortality were made at 24, 48, 72, 96, and 120 hr. (Vimala Devi and Vineela, 2015 c). An untreated heck was also kept in order to obtain corrected mortality. Using the common formula, the mortality % was calculated in accordance with Rajamanickam et al. (2015). In surface contamination method, cell culture supernatant was overlayed on the semisynthetic diet and it was kept in the room temperature till the overlayed supernatant was dried (Vimaladevi and Vineela, 2015).

For standardization of concentration of promising isolates of *B. thuringiensis* comparing the reference strain *B. thuringiensis* HD1 (Dr P U Krishnaraj, IABT,UAS, Dharwad). Isolates that recorded more than 20% were further tested at various concentrations. Cell culture supernatant of *B. thuringiensis* isolates was diluted with a semisynthetic diet at five concentrations ($1x10^5$, $2x10^5$, $2.5x10^5$, $3x10^5$ and $3.5x10^5$ ppm) for conducting bioassay. As a control, larvae were fed with sterile Luria broth media incorporated into their diet. The procedure followed in the preliminary bioassay was also followed in standardization of concentration. The median lethal concentration was determined using concentrations and mortality data ($LC_{50}$). The data were subjected to statistical analysis using completely randomized design (CRD). Duncan's multiple range test (DMRT) was used to separate means after the data had undergone arcsine transformation and had undergone analysis of variance. Calculated median lethal concentration ($LC_{50}$) using probit analysis.

To identify the insecticidal toxic protein genes (*Vip*, *Cyt*), all the *B. thuringiensis* isolates were used in insecticidal activity were used to extract genomic DNA and amplify (*Vip*, *Cyt*) genes by PCR technique. The total genomic DNA was isolated by following the protocol of Sambrook and Russel (2001) with some modifications in the incubation time and temperature. Eppendorf tubes containing bacterial pellet were treated with lysozymes. Further tubes were treated with 5 µl of RNase (10mg/ml), vertexed and incubated at 37ºC for 1.5 hr again. Later, 50 µl of 10% SDS and 5 µl proteinase (20 mg/ ml) were added, vortexed for 20 seconds and incubated at 37ºC for 20 minutes. Purity and yield of the extracted DNA were assessed spectrophotometrically using the nanodrop method (Thermo scientific- Nanodrop 2000) and calculated $A_{260}$/ $A_{280}$ ratios to determine protein impurities and DNA concentrations.

To detect the presence of the insecticidal toxin genes listed in Table 1, polymerase chain reaction was used with specific primers. Fifteen (native and NCIM) strains of *B. thuringiensis* used in bioassay were used for insecticidal toxin protein gene profiling. Additional eighteen UASR-MLK *B. thuringiensis*

Table 1. Preliminary evaluation of cell supernatant of native and NCIM isolates of *B. thuringiensis* against second instar larvae of *S. litura* (diet incorporation method)

| Sl. No. | Isolates | % mortality of *S. litura* larvae | | | | |
|---|---|---|---|---|---|---|
| | | 1 DAT* | 2 DAT | 3 DAT | 4 DAT | 5 DAT |
| 1 | BGC-1 | 0.00 (0) [d] | 3.33 (10.49) [d] | 6.66 (14.95) [d] | 10.00 (18.43) [d] | 13.33 (21.41) [d] |
| 2 | BGC-2 | 0.00 (0) [d] | 0.00 (0) [e] | 3.33 (10.49) [e] | 6.66 (14.92) [e] | 10.00 (18.40) [e] |
| 3 | RCM-2 | 0.00 (0) [d] | 0.00 (0) [e] | 3.33 (10.49) [e] | 6.66 (14.92) [e] | 10.00 (18.40) [e] |
| 4 | GBP-2 | 0.00 (0) [d] | 0.00 (0) [e] | 3.33 (10.49) [e] | 6.66 (14.92) [e] | 10.00 (18.40) [e] |
| 5 | GHB-1 | 0.00 (0) [d] | 0.00 (0) [e] | 3.33 (10.49) [e] | 6.66 (14.92) [e] | 6.66 (14.92) [f] |
| 6 | MDH | 0.00 (0) [d] | 0.00 (0) [e] | 3.33 (10.49) [e] | 6.66 (14.92) [e] | 10.00 (18.40) [e] |
| 7 | KMS-2 | 0.00 (0) [d] | 3.33 (10.49) [d] | 6.66 (14.92) [d] | 10.00 (18.40) [d] | 13.33 (21.37) [d] |
| 8 | GHM-1 | 0.00 (0) [d] | 0.00 (0) [e] | 3.33 (10.49) [e] | 6.66 (14.92) [e] | 10.00 (18.40) [e] |
| 9 | GHM-2 | 0.00 (0) [d] | 0.00 (0) [e] | 3.33 (10.49) [e] | 6.66 (14.92) [e] | 10.00 (18.40) [e] |
| 10 | MDS-2 | 0.00 (0) [d] | 0.00 (0) [e] | 3.33 (10.49) [e] | 6.66 (14.92) [e] | 10.00 (18.40) [e] |
| 11 | HD-1 | 0.00 (0) [d] | 3.33 (10.49) [d] | 6.66 (14.92) [d] | 10.00 (18.40) [d] | 13.33 (21.37) [d] |
| 12 | NCIM-5111 | 13.33 (21.37) [a] | 16.66 (24.04) [a] | 20.00 (26.51) [a] | 23.33 (28.82) [a] | 26.66 (31.02) [a] |
| 13 | NCIM-5112 | 6.66 (14.92) [c] | 10.00 (18.43) [c] | 13.33 (21.41) [c] | 16.66 (24.09) [c] | 20.00 (26.56) [c] |
| 14 | NCIM-5116 | 10.00 (18.40) [b] | 13.33 (21.37) [b] | 16.00 (23.53) [b] | 20.00 (26.51) [b] | 23.33 (28.82) [b] |
| 15 | NCIM-5117 | 6.66 (14.92)[c] | 10.00 (18.43) [c] | 13.33 (21.41) [c] | 16.66 (24.09) [c] | 20.00 (26.56) [c] |
| 16 | Control | 0.00 (0) [d] | 0.00 (0) [e] | 0.00 (0) [f] | 0.00 (0) [f] | 0.00 (0) [g] |
| S.Em± | | 0.105 | 0.143 | 0.188 | 0.248 | 0.307 |
| CD (p= 0.01) | | 0.303 | 0.415 | 0.544 | 0.717 | 0.888 |

Figures in parentheses arcsine transformed values; Values represented by same alphabet statistically on par with each other by (DMRT); DAT*- Days after treatment.

(UASR-MLK-*Bt*-21, *Bt*-22, *Bt*-24, *Bt*-25, *Bt*-26, *Bt*-27, *Bt*-28, *Bt*-29, *Bt*-33, *Bt*-36, *Bt*-41, *Bt*-42, *Bt*-43, *Bt*-44, *Bt*-46, *Bt*-47, *Bt*-48, *Bt*-49) isolates were also screened by colony PCR for the presence of insecticidal toxin genes. According to Rabha et al. (2017), PCR analysis of the *B. thuringiensis* isolates was performed with slight modifications in the specific primer pairs, template DNA, annealing temperature, concentration of the reagents and number of cycles used in the PCR analysis. By PCR amplification, set of specific primers and insecticidal toxin protein genes were analyzed from 32 *B. thuringiensis* isolates and one reference strain (*B. thuringiensis var kurstaki* HD-1). Each samples reaction mixture was made separately and distributed into 33 tubes of 0.2 ml PCR tubes (18µl/tube), to which 2µl of template DNA was added to bring the total reaction volume to 20µl. For each reaction, 20µl of PCR reaction or master mix was prepared by adding 10µl of Taq mix (2X), 2µl of 10 pM primer (1µl forward primer + 1µl reverse primer), 2µl of template DNA or colony from the respective isolates of *B. thuringiensis* and 6µl of nuclease free water was added to make the total volume to 20µl and PCR was carried out in gradient thermal cycler (Takara). PCR cycling for these reactions was as follows: 3 min initial denaturation at 95°C followed by 30 cycles of amplification with 1 min denaturation at 95°C, 1 min of annealing at 56.2°C (*Vip*1), 57.9°C (*Vip*1-sc.), 49°C (*Vip*1-tp.), 48.9°C (*Vip*2-sc.), 48.7°C (*Vip*2-tp.), 52-55°C (*Vip*3), 59.4°C (Cyt1) and 45°C (Cyt2) and 45-60 sec of initial extension at 72°C. An extra extension step of 10 min at 72°C and hold of amplified PCR reactions at 4°C was followed after completion of amplification. Specific primers used in the current study were 1) *Vip*1 (#26) FP : AAATTAGTGATCCGTTACCTTCTT, (#27) (742bp) RP: AACTTGCTTTTCTTTCCCTTTAT (Shingote et al., 2013); 2) *Vip*1-sc (#30) ATAAAGAAAAAGCAAAAGAATGGG RNAA RRA,(#31)ACCATCTATATACAGTAATATTTT CTGGDATNGG (585bp) (Rodriguez et al., 2009) ; 3) *Vip*1-type (#32) FP: AAACGGGTGATTTYACNTT (#33) RP: GGGAATTAAAATCATCCAT (1044 bp) (Rodriguez et al., 2009); 4) *Vip*2-sc (#30) FP: GATA AAGAAAAAGCAAAAGAATGGGRNAARRA(#31) RP:CCACACCATCTATATAC

AGTAATATTTTCTGGDATNGG (845bp) (Rodriguez et al., 2009); 5) *Vip*2-tp (#34) FP: AGAATGGGGGAA AGARAA (#35) RP: ACCTCTGTTACTTT ATCDAT (1107bp)

(Rodriguez et al., 2009) ; 6) *Vip*3 (#12) FP:TTATTTTAATGGCATTTATGGATTTGCC (#13) RP:GCAGGTGTAATTTCAGTAAGTGTAGAG (444bp) (Fang et al., 2007); 7) *Vip*3 (#14)FP: CTTCTGAAAAGTTATTAAGTCCAGAA (#15) RP: TTACTTAAT AGAGACATCGTAAAAA (Fang et al., 2007); (364bp) 8) Partial Cyt1 genes (#44) FP:AACCCCTCAATCAACAGCAAGG (#45) RP:GGTACACAATACATAACGCCACC (522-525 bp) (Jain et al., 2017); 9) Partial Cyt2 genes (#46)FP:AATACATTTCAAGGAGCTA(#47) RP:TTTCATTTTAACTTCATATC (469 bp) (Jain et al., 2017). The toxins Vip1 and Vip2 are Coleopteran and Hemipteran specific (Binary toxins), Vip3 are Lepidopteran specific and Cyt 1 and Cyt2 are Dipteran specific toxins. The PCR products were examined by agarose gel electrophoresis. The PCR products obtained after amplification were scored for the presence or absence of the different insecticidal toxin genes mentioned in Table 1. Amplified fragments were purified with the HiPer gel extraction teaching kit (HTBM010) and submitted for sequencing. The toxin gene sequences were aligned with MEGA 6.0 version software and it was also used for the construction of an unrooted phylogenetic tree. Further Chimera 2.0 parameter was used with 1000 boot troops to construct the maximum likelihood phylogenetic tree.

## RESULTS AND DISCUSSION

Preliminary evaluation of cell culture supernatant of native and NCIM *B. thuringiensis* isolates against *S. litura* was carried out. In diet incorporation ($1x10^5$ ppm) method, *S. litura* mortality generally increased with incubation period, reaching maximum percent mortality after five days of feeding. Among the fifteen *B. thuringiensis* isolates, only four NCIM (5111, 5112, 5116 and 5117) isolates caused 20.0 to 26.66% mortality remaining eleven isolates (BGC-1, BGC-2, RCM-2, GBP-2, MDH, KMS-2, GHM-1, GHM-2, MDS-2, HD-1 and GHB-1) were exhibited below 13.33 % of mortality against second instar larvae of *S. litura* (Table 1). The larval mortality in the experiment generally ranged from 0.00 to 13.33% up to one day. But increased with increase in the time. Finally, at five days after exposure, the maximum mortality was recorded between 6.66 to 26.66%. When an insect ingests active component of the cell supernatant such as vegetative insecticidal proteins (*Vip*), which results in epithelial cells lysis and feeding activity being paralyzed, which ultimately causes the insect to die from starvation and septicemia (Chakroun et al., 2016). Above results were in concurrence with the similar type of studies by Sattar et al. (2008) and Sahin et al. (2018). In surface contamination method, out of the fifteen *B. thuringiensis* isolates screened, only four NCIM isolates (5111, 5112, 5116 and 5117) noticed mortality above 23.33%, remaining eleven isolates (BGC-1, BGC-2, RCM-2, GBP-2, GHB-1, MDH, KMS-2, GHM-1, GHM-2, MDS-2 and HD-1) observed mortality below 10% against second instar larvae of *S. litura* (Table 2). These results are in conformity with the reports of Palma et al. (2013) and Lone et al. (2016).

The range of the median lethal concentration ($LC_{50}$) of promising isolates was ranged from $2.5x10^5$ to $3.33x10^5$ ppm in the diet incorporation method. The $LC_{50}$ value of NCIM-5111 isolate was found to be the lowest with $2.5x10^5$ ppm, which was comparable to the $LC_{50}$ value of NCIM-5116 isolate ($2.64x10^5$ ppm). This was followed by the NCIM-5112 isolate with $LC_{50}$ value of $2.79x10^5$ ppm and NCIM-5117 isolate with $LC_{50}$ value of $3.33x10^5$ ppm concentration (Table 3). Generally, the median lethal concentration ($LC_{50}$) of promising isolates using the surface contamination method was ranged from $2.20x10^5$ to $2.85x10^5$ ppm. The $LC_{50}$ value f NCIM-5111 isolate was found to be the lowest with $2.20x10^5$ ppm. This was followed by the NCIM-5116 isolate with $LC_{50}$ value of $2.3x10^5$ ppm and NCIM-5112 isolate with $LC_{50}$ value of $2.51x10^5$ ppm which was compared to the $LC_{50}$ value of NCIM-5117 isolate with $2.85x10^5$ ppm (Table 3). The results indicated that as the cell supernatant concentration increases, the mortality also increases in all the isolates. But there was decrease in mortality rate as the increase in the age of the larvae noticed in all the isolates. This is may be because of susceptibility of early instar larvae to toxins than the older or later age larvae. The results of our present findings are well supported by the results of earlier studies (Sattar et al., 2008; Song et al., 2016; Sahin et al., 2018; Guney et al., 2019). The lowest $LC_{50}$ was observed in the isolate NCIM-5111, which was found to be relatively effective. The higher efficacy of the isolate NCIM-5111 may be due to higher concentration of *Vip* protein secretion or quality of *Vip* proteins. The binding of these proteins results in the formation of ion channels that are distinct from those formed by *Cry1Ab*. These toxicity differences could be attributed to differences in the source of the insect strain used for testing (Martinez et al., 2013) or such differences in the insecticidal activity of different isolates of *B. thuringiensis* might be with deletions of some amino acids at the initial N-terminal end and also could be due to the higher concentration of the *Vip3* protein in the strain (NCIM-5111). The N-terminal

Table 2. Preliminary evaluation of native and NCIM isolates of *B. thuringiensis* against second instar larvae of *S. litura* (surface contamination method)

| Sl. No. | Isolates | % mortality of *S. litura* larvae | | | | |
|---|---|---|---|---|---|---|
| | | 1 DAT* | 2 DAT | 3 DAT | 4 DAT | 5 DAT |
| 1 | BGC-1 | 0.00 (0.00) [d] | 3.33 (10.49) [e] | 6.66 (14.95) [e] | 13.33 (21.37) [d] | 16.66 (23.53) [e] |
| 2 | BGC-2 | 0.00 (0.00) [d] | 3.33 (10.49) [e] | 6.66 (14.95) [e] | 10.00 (18.40) [e] | 13.33 (21.37) [f] |
| 3 | RCM-2 | 0.00 (0.00) [d] | 3.33 (10.49) [e] | 6.66 (14.95) [e] | 10.00 (18.40) [e] | 13.33 (21.37) [f] |
| 4 | GBP-2 | 3.33 (10.49) [c] | 3.33 (10.49) [e] | 6.66 (14.95) [e] | 10.00 (18.40) [e] | 13.33 (21.37) [f] |
| 5 | GHB-1 | 0.00 (0.00) [d] | 0.00 (0.00) [f] | 3.33 (10.49) [f] | 6.66 (14.95) [f] | 10.00 (18.40) [g] |
| 6 | MDH | 0.00 (0.00) [d] | 3.33 (10.49) [e] | 6.66 (14.95) [e] | 10.00 (18.40) [e] | 13.33 (21.37) [f] |
| 7 | KMS-2 | 3.33 (10.49) [c] | 6.66 (14.95) [d] | 10.00 (18.40) [d] | 13.33 (21.37) [d] | 16.66 (23.53) [e] |
| 8 | GHM-1 | 0.00 (0.00) [d] | 3.33 (10.49) [e] | 6.66 (14.95) [e] | 10.00 (18.40) [e] | 13.33 (21.37) [f] |
| 9 | GHM-2 | 0.00 (0.00) [d] | 3.33 (10.49) [e] | 6.66 (14.95) [e] | 10.00 (18.40) [e] | 13.33 (21.37) [f] |
| 10 | MDS-2 | 0.00 (0.00) [d] | 3.33 (10.49) [e] | 6.66 (14.95) [e] | 10.00 (18.40) [e] | 13.33 (21.37) [f] |
| 11 | HD-1 | 3.33 (10.49) [c] | 6.66 (14.95) [d] | 10.00 (18.40) [d] | 13.33 (21.37) [d] | 16.66 (23.53) [e] |
| 12 | NCIM-5111 | 16.66 (23.53) [a] | 20.00 (26.56) [a] | 23.33(28.88) [a] | 30.00 (33.21) [a] | 33.33 (35.26) [a] |
| 13 | NCIM-5112 | 10.00 (18.40) [b] | 13.33 (21.37) [c] | 16.66 (23.53) [c] | 23.33 (28.88) [b] | 26.66 (31.09) [c] |
| 14 | NCIM-5116 | 10.00 (18.40) [b] | 16.66 (23.53) [b] | 20.00 (26.56) [b] | 23.33 (28.88) [b] | 30.00 (33.21) [b] |
| 15 | NCIM-5117 | 10.00 (18.40) [b] | 13.33 (21.37) [c] | 16.66 (23.53) [c] | 20.00 (26.56) [c] | 23.33 (28.88) [d] |
| 16 | Control | 0.00 (0.00) [d] | 0.00 (0.00) [f] | 0.00 (0.00) [g] | 0.00 (0.00) [g] | 0.00 (0.00) [h] |
| S.Em± | | 0.103 | 0.153 | 0.222 | 0.304 | 0.363 |
| CD @1% | | 0.297 | 0.443 | 0.641 | 0.879 | 1.049 |

Figures in parentheses arcsine transformed values; Values represented by same alphabet statistically on par with each other (DMRT); DAT*- Days after treatment.

region of *Vip*3A proteins has a highly conserved amino acid sequence, which suggests that this region probably plays a significant role in protein structure and insecticidal activity (Li et al., 2007). Comparison of % mortality of the two methods of bioassay reveals that there was increase in the mortality from 6.66 to 10.00% (surface contamination method) across the isolates NCIM-5111, NCIM-5112, NCIM-5116 and NCIM-5117 (Table 3). However, comparison of % mortality between the two methods of bioassay was found non-significant.

Thirty-three *B. thuringiensis* strains were screened for the presence of *Vip* and *Cyt* genes using specific screening primers. Among them, only one *B. thuringiensis* isolate (NCIM-5112) was found positive to carry *Vip1* gene with an expected band size of 742bp and the same isolate produced a light amplification product (weak bands) of unexpected size. The amplified product of *Vip*1 specific primer was designed based on two known sequences of genes coding for *Vip1Aa/Vip2Aa* and *Vip1Ab/Vip2Ab7*. Forward primer corresponds to positions 1076–1099bp and the reverse primer corresponds to positions 1794–1817bp of *Vip1Ab/Vip2Ab* sequence. The amplified 742bp fragment contained the 3-terminus of the *Vip2* gene and the 5-terminus of the *Vip1* gene. It showed a significant difference from all existing *Vip* genes. This indicates the obtained *Vip1/Vip2* gene has different motifs than the available *Vip* toxins. Similar results were reported by Shingote et al. (2013). Among 33 strains, only four strains NCIM-5111, NCIM-5112, NCIM-5116 and NCIM-5117 were successfully amplified the *Vip3* gene for both primers with a DNA fragments size of 444 and 364bp, respectively. The present results in concurrence with those of Fang et al. (2007), who amplified two DNA fragments from bacterial isolates indicated the presence of a *Vip3* gene, which is highly similar to gene *Vip3Aa1*. The PCR products obtained after amplification were subjected for gel electrophoresis and amplified fragments were eluted and submitted for sequencing. The toxin gene sequences were further confirmed by the NCBI-BLAST analysis of partial genome sequence (100.00% similarity), amplified sequences were aligned with MEGA 6.0 version software and it was also used for the construction of an unrooted phylogenetic tree by considering the most reference sequences from the NCBI along with sequences of *Vip*3, *Vip*1 to study the evolutionary relationship between different isolates. Further Chimera 2.0 parameter was used with 1000

Table 3. $LC_{50}$ of potential *B. thuringiensis* isolates against *S. litura* in the diet incorporation and surface contamination method

| Treatment details | Diet incorporation method | | | | | Surface contamination method | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | % mortality of *S. Litura* 5DAT @ ($3.5x10^5$ spores/ ml) | $LC_{50}$ (ppm) | Fiducial limit | | Regression equation | $\chi 2$ value | % mortality of *S. Litura* 5DAT @ ($3.5x10^5$ spores/ml) | $LC_{50}$ (ppm) | Fiducial limit | | Regression equation | $\chi 2$ value |
| | | | Lower limit | Upper limit | | | | | Lower limit | Upper limit | | |
| NCIM-5111 | 56.66 (48.83) | $2.5x10^5$ | $2.29x10^5$ | $2.74x10^5$ | Y= -30.3471 + 6.54641x | 5.99 | 66.66 (54.73) | $2.20x10^5$ | $2.02x10^5$ | $2.39x10^5$ | Y= -31.7424 + 6.87973x | 5.99 |
| NCIM-5112 | 50.00 (45.00) | $2.79x10^5$ | $2.52x10^5$ | $3.07x10^5$ | Y= -30.8257 + 6.57995x | 5.99 | 56.66 (48.83) | $2.51x10^5$ | $2.30x10^5$ | $2.75x10^5$ | Y= -30.3470 + 6.54636x | 5.99 |
| NCIM-5116 | 53.33 (46.91) | $2.64x10^5$ | $2.40x10^5$ | $2.90x10^5$ | Y= -30.5859 + 6.56307x | 5.99 | 63.33 (52.73) | $2.3x10^5$ | $2.12x10^5$ | $2.5x10^5$ | Y= -31.9812 + 6.89643x | 5.99 |
| NCIM-5117 | 36.66 (37.26) | $3.33x10^5$ | $2.88x10^5$ | $3.88x10^5$ | Y= -24.5301 + 5.34610x | 5.99 | 46.66 (43.08) | $2.85x10^5$ | $2.55x10^5$ | $3.18x10^5$ | Y= -27.8432 + 6.02141x | 5.99 |
| Mean | 49.16 (44.52) | | | | | | 58.32 (49.79) | | | | | |

Figures in parentheses arcsine transformed values; DAT*- Days after treatment

boot troops to construct the maximum likelihood phylogenetic tree.

Phylogenetic analysis of *B. thuringiensis* strains showing *Vip3* gene using primer (#12, #13) was established. The strains belonging to NCIM formed a separate cluster and the rest of the species formed a cluster excluding NCIM isolates. Two isolates of NCIM, PuneNCIM-5111 (#12, #13) strain and NCIM-5116 (#12, #13) strain formed one subgroup with bootstrap value 100-61 % and the other two isolates of NCIM, Pune, India, NCIM-5112 (#12, #13) strain; NCIM-5117 (#12, #13) strain formed one subgroup with bootstrap value 100-31% this indicated that they are strongly supported (Fig. 1). Phylogenetic analysis of *B. thuringiensis* strains showing *Vip3* gene using primer (#14, #15) was carried out. *B. thuringiensis* isolates could be divided into two primary clusters. NCIM isolates formed a distinct cluster and the rest of the species formed a different cluster. Two isolates of NCIM viz., NCIM-5111 (#14, #15) strain and NCIM-5116 (#14, #15) strain formed one subgroup with bootstrap value 100-64 percent and the other two NCIM isolates, NCIM-5112 (#14, #15) strain and NCIM-5117 (#14, #15) strain formed one subgroup with bootstrap value 100-38 percent, indicating that they are strongly supported (Fig. 2). Phylogenetic analysis of *B. thuringiensis* strains showing *Vip1* gene using (#26, #27) primer was established. *B. thuringiensis Vip*2Ac (*Vip2Ac*) and *Vip*1Ac (*Vip1Ac*) formed a distinct cluster and the rest of the species formed another cluster that include isolate NCIM-5112 (#26, #27). *B. thuringiensis* strain HBF-18 *Vip*1 (*Vip1*), *B. thuringiensis* strain 376 (*Vip1*), *B. thuringiensis* isolate DGA9 (*Vip1*), *B. thuringiensis* strain SVBS18-01, *B. thuringiensis* strain 376 (*Vip2*) and NCIM-5112 (#26, #27) strain produced a subgroup with bootstrap value of 61-84 % which implies that they are strongly supported (Fig. 3). Thus, four NCIM isolates (5111, 5112, 5116 and 5117) were found to have potential, and caused highest mortality of second instar larvae of *S. litura.* Among four isolate NCIM-5111 showed the least $LC_{50}$ value of $2.5x10^5$ ppm in the diet incorporation and $2.20x10^5$ ppm in the surface contamination method. Among thirty-three *B. thuringiensis* isolates screened for *Vip* and *Cyt* genes, only four NCIM isolates (12.12%) were found to contain *Vip3* genes.

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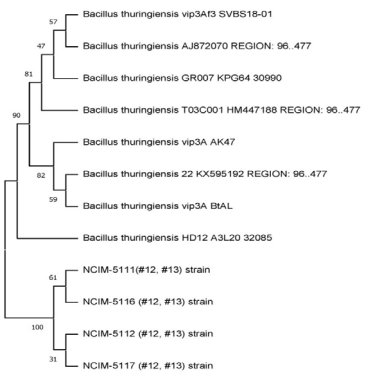


Fig. 1. Phylogenetic study of *B. thuringiensis* strains expressing the *Vip3* gene using primers (#12, #13)

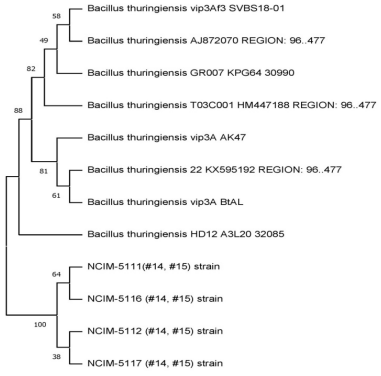


Fig. 2. Phylogenetic study of *B. thuringiensis* strains employing primers (#14, #15) to screen the *Vip3* gene

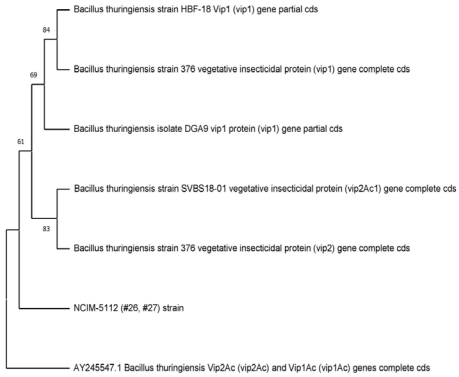


Fig. 3. Phylogenetic study of *B. thuringiensis* strains using primers (#26, #27) to screen the *Vip*1 gene

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## AUTHOR CONTRIBUTION STATEMENT

Basavaraj S Kalmath and Lakshmikanth conceptualized and framed the research proposal. Mahadev Naik conducted the experiment, curated data and prepared original draft. Saroja Rao supported for technical suggestions. Syed Dastager has given four isolates of *B. thuringiensis*. Harischandra Naik has supported financially to carry out experiments. Aarunkumar Hosamani and Basavaraj Kadannavar contributed to the samples, analyzed the results and corrected draft. All authors read and approved the manuscript.

## CONFLICT OF INTEREST

No conflict of interest.

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