1 **Molecular and insecticidal characterization of Vip3A protein producing *Bacillus***
2 ***thuringiensis* strains toxic against *Helicoverpa armigera* (Lepidoptera: Noctuidae)**

3 **Showkat Ahmad Lone^{1,2}, Radha Yadav², Abdul Malik,¹ Jasdeep Chatrath Padaria^{2*}**

4 ¹Department of Agricultural Microbiology, Faculty of Agricultural Sciences, Aligarh Muslim
5 University, Aligarh, Uttar Pradesh, India-202002

6 ²Biotechnology and Climate Change Group, National Research Centre on Plant
7 Biotechnology, Pusa Campus, New Delhi, India-110012

8 *E-mail: jasdeep_kaur64@yahoo.co.in

9 Tel.: +91-011-25843201 (ext.270); Fax: +91-011-25843984

Draft

24 **Abstract**

25 Vegetative insecticidal proteins (Vip) represent the second generation of insecticidal proteins
 26 produced by *Bacillus thuringiensis* (Bt) during the vegetative growth stage of growth. Bt
 27 based biopesticides are recognized as viable alternatives to chemical insecticides, the latter
 28 cause environmental pollution and lead to emergence of pest resistance. In order to perform a
 29 systematic study of *vip* genes encoding toxic proteins a total of 30 soil samples were
 30 collected from diverse locations of Kashmir valley, India and characterized by molecular and
 31 analytical methods. 86 colonies showing *Bacillus* like morphology were selected. Scanning
 32 Electron Microscopy observations confirmed the presence of different crystal shapes, PCR
 33 analysis of insecticidal genes revealed predominance of lepidopteran specific *vip3* (43.18%)
 34 followed by coleopteran specific *vip1* (22.72%) and *vip2* (15.90%) genes in the isolates
 35 tested. Multi-alignment of the deduced amino acid sequences revealed that *vip3* sequences
 36 were highly conserved whereas *vip1* and *vip2* showed adequate differences in amino acid
 37 sequences compared to already reported sequences. Screening for toxicity against
 38 *Helicoverpa armigera* larvae was performed using partially purified soluble fractions
 39 containing Vip3A protein, mortality levels observed ranged between 70% and 96.6% in the
 40 isolates. LC50 values of two of the native isolates JK37 and JK88 against *H. armigera* was
 41 found to be at par with that of Bt *kurstaki* HD1, suggesting that these isolates could be
 42 developed as effective biopesticides against *H. armigera*.

43 **Keywords:** *Bacillus thuringiensis*, scanning electron microscopy (SEM), Vip proteins,
 44 toxicity assay, *Helicoverpa armigera*.

45

46

47

48

49

50 Introduction

51 Microbial insecticides are effective alternatives to chemical insecticides for the control of
52 various insect pests. Their greatest strengths are safety to humans and other non- target
53 groups, specificity against target insects and no hazardous residues left after treatment. One
54 of the most successful microbial alternatives to chemical insecticides is entomopathogenic
55 bacterium, *Bacillus thuringiensis* (Bt). Bt is known for its ability to produce protein crystals
56 with insecticidal properties. These toxins are widely used for the control of various
57 agricultural pests due to their high specificity and environmental friendly nature. Bt, in
58 addition to crystalline proteins (Cry and Cyt) which are produced during sporulation also
59 produces a relatively novel class of insecticidal proteins called Vegetative Insecticidal
60 Proteins (Vips) during vegetative stage of growth (Warren et al. 1996). Four classes of these
61 proteins have been reported so far; Vip1, Vip2, Vip3 and Vip4. Vip1 and Vip2 are binary
62 toxins effective against coleopteran pests, whereas Vip3 toxins are effective against
63 lepidopteran pests (Estruch et al. 1996). Presently, there are 107 Vegetative Insecticidal
64 Protein coding gene sequences known, out of which 12 sequences are *vip1*, 17 *vip2*, 77 *vip3*
65 and 1 sequence *vip4* (Crickmore 2014). These toxins are classified on the basis of degree of
66 amino acid homology (http://www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/vip.html).
67 Vip proteins represent the second generation of insecticidal trans-genes, and offer broad
68 spectrum of activity against insect pests. The difference in amino acid homology and mode of
69 action of Vip toxins from that of crystalline protein toxins makes them good candidates to
70 overcome insect resistance development (Bravo et al. 2011).

71 Cry proteins have been extensively used for the control of various agriculturally important
72 pests including the infamous cotton bollworm *Helicoverpa armigera*. However, there are
73 several reports of field evolved resistance in *H. armigera* against Cry toxins throughout the
74 world (Van Rensburg 2007; Dhurua and Gujar 2011) raising concerns about the adequacy of

the current resistance management strategies (Gassmann et al. 2014). In order to improve the prospects for insect pest control more Vip toxin proteins must be explored from *B. thuringiensis* strains isolated from relatively unexplored environments, as such environments may contain novel isolate(s) having toxicity against a particular class of insect pests. There are various reports on isolation of novel *B. thuringiensis* strains from the various environments (Vilas-Boas and Lemos 2004; Lone et al. 2014). Most of these studies have focussed on diversity of crystalline coding genes (*cry* followed by *cyt*) using PCR based approach. On the contrary, there have been few reports on the diversity of *vip* genes in *B. thuringiensis* from different regions of the world (Beard et al. 2008; Yu et al. 2011). Diversity and distribution of *vip* genes of Bt in some geographical regions of India has been investigated earlier (Sattar and Maiti 2011; Shingote et al. 2013). However there has been no report on such studies in Kashmir Valley. The unique geomorphology of the valley provides an opportunity to isolate novel Bt strains carrying novel vegetative insecticidal protein (Vip) coding genes. Therefore, a detailed study is needed to assess the genetic diversity among Bt strains of the valley and to study the distribution patterns of *vip* genes in these isolates. The present study was carried out with the aim of studying the diversity and distribution of *B. thuringiensis* with respect to the *vip* gene content they possess and to enrich the available resources with novel insecticidal Vip proteins and more efficient insecticidal strains for the control of lepidopteran pests. The Bt strains were isolated from ten locations in Kashmir Valley and characterized by SEM observation of crystalline proteins, sequence analysis of 16S ribosomal RNA gene, PCR based screening for the presence of Vegetative insecticidal protein coding genes (*vips*) and the toxicity screening against a polyphagous lepidopteran pest *H. armigera*.

Materials and methods

Sample collection

A total of 30 soil samples were collected from 10 different regions of Kashmir Valley; regions unaffected by anthropogenic activities were selected (Table 1). There was no previous history of use of *B. thuringiensis* or its products in the soil sampling areas. The soil samples were collected at a depth of 10 cm from the surface and transported to the laboratory, so that the elapsed time between sample collection and initial processing did not exceed 24 h for the isolation of *B. thuringiensis*.

Bacillus thuringiensis strains

Sodium acetate enrichment method (Travers et al. 1987) was used for isolation of *B. thuringiensis* from the collected soil samples. For each plated sample, well isolated colonies representing *Bacillus* like morphology were picked up and purified on T3 agar plates containing Penicillin at a concentration of 10 $\mu\text{g mL}^{-1}$. Bt index was calculated as suggested by Baig et al. (2010). The standard *B. thuringiensis* strain (*Bt kurstaki* HD1) used as reference for the insect bioassays was kindly provided by Dr. Daniel R. Zeigler from the *Bacillus* Genetic Stock Center (Ohio, USA).

Phenotypic identification

Bacillus isolates were inoculated in 50 mL Luria broth in 250 mL Erlenmeyer flasks and incubated at 30 °C for 72 h with shaking at 250 rpm. The autolysed cells were harvested to obtain spore-crystal mixture by centrifuging at 12,000 g at 4 °C for 20 min. The pellets were washed in 0.5 M NaCl thrice followed by washing thrice in distilled water to eliminate extra cellular components. The pellets so obtained, were finally resuspended in sterile distilled water. The presence of parasporal crystals was observed under phase contrast microscope. In order to confirm the crystal shape, Scanning Electron Microscopy (SEM) was performed as, another set of autolysed cell pellets were washed with Phosphate buffer saline (PBS) thrice followed by fixing the pellet in 2.5% glutaraldehyde overnight at 4 °C. Glutaraldehyde was removed by washing the pellet thrice with PBS followed by washing through ethanol

gradients (10% through 30%, 70% and absolute). The pellets were suspended in 20 µl sterile distilled water and mounted on brass stub, air dried, sputter-coated with gold and then examined under SEM (JSM6610lv Jeol, Japan).

16S rRNA gene sequencing and phylogenetic tree construction

PCR amplification of 16S rRNA gene from the selected *Bacillus* isolates was performed using the universal primers: forward (27f) 5'-AGAGTTTGATCCTGGCTCAG-3', reverse (5210r) 5'-AAGGAGCTGATCCAGCCGCA-3'. The purified amplicons of 16S rDNA gene were sequenced using primers 27f and 5210r with fluorescent terminators (Big Dye, Applied Bio systems). The homology of the sequences obtained were determined by NCBI BLAST (Altschul et al. 1990). Sequences were aligned, verified and edited using Bioedit 7.2.0 (Hall 1999) the phylogenetic tree based on 16S rRNA sequences was constructed using MEGA v6.0 (Tamura et al. 2013) by Neighbor joining (NJ) (Nei and Kumar 2000) method. Gaps were considered missing data points, genetic distances were estimated using nucleotide/Jukes-Cantor (for rRNA) (Jukes and Cantor 1969) and the statistical significance of the branching order of the tree was established by bootstrap analysis using 1000 permutations of the data set.

Identification of *vip* genes

For detection of *vip* gene combinations, all the native *B. thuringiensis* isolates were subjected to PCR analysis using 3 pairs of *vip* gene specific primers listed in Table 2. Two of the primers (Vip1 smp and Vip2 smp) are degenerate and were designed in this study, while as the third primer (Vip3A) was taken from a published study (Selvapandiyan et al. 2001). The Vip1 primers were designed from the conserved regions of *vip1Aa2*, *vip1Ac*, *vip1Ba1*, *vip1Bb1*, and *vip1Da1* (Acc. Nos. AAR81088, AAO86514, AAR40886, AAR40282, AAT21728 respectively); Vip2 primers from conserved regions *vip2Aa2*, *vip2Ac1*, *vip2Ad1*, *vip2Af1*, and *vip2Ba1* (Acc. Nos. AAR81096, AAO86513, AAT21729, ACH42759,

AAR40887 respectively) and Vip3 primers from *vip3Aa* and *vip3Ab* sequences (Selvapandiyan et al. 2001). PCR reactions were performed using 100 ng of total *B. thuringiensis* DNA with 0.6 mM dNTP mix, 0.5 μ M each primer, 3 mM MgCl₂, 1.5 U of Taq DNA polymerase (Fermentas, USA) in a final volume of 25 μ l. Amplification was performed at following conditions: 94 °C for 1 min, annealing at 54-55 °C (Table 2) for 1 min and extension at 72 °C for 1 min. An extra step of extension at 72 °C for 10 min was added after completion of 25 cycles. PCR products were sequenced (Big Dye, Applied Bio systems) using the same primers that were used to generate PCR products.

Protein purification

Vegetative insecticidal proteins from native and reference *B. thuringiensis* strains were purified from culture supernatants by the method described by Estruch et al. (1996). The cultures were grown for 16 h at 30 °C in Terrific broth (12% tryptone, 2.4% yeast extract, 0.04% glycerol, 0.17 M KH₂PO₄, 0.72 M K₂HPO₄) and centrifuged at 5000 x g for 20 min. The supernatant containing proteins was precipitated with ammonium sulfate (70% (w/v) saturation) and proteins were collected by centrifugation at 5000 x g for 15 min. The pellets thus obtained were resuspended in the original volume of 20 mM Tris HCl (pH 7.5) and dialyzed overnight at 4 °C followed by titration to pH 4.5 using 20 mM sodium citrate (pH 2.5). After 30 min incubation at room temperature, the samples were centrifuged at 3000 x g for 10 min. The protein pellets were washed once with 1 mL⁻¹ NaCl containing 5 mL⁻¹ EDTA followed by resuspension in 0.02 M Tris, pH 8 and filtered (0.2 μ m).

Helicoverpa culture

H. armigera larvae were collected between March 2013 to May 2013 from infested chick pea plants grown at the experimental farms of faculty of agricultural sciences, Aligarh Muslim University, Aligarh, India. The insects were maintained in our laboratory for 10 generations without exposure to insecticides before proceeding with bioassay and toxicity experiments.

Helicoverpa larvae were initially maintained on natural diet consisting of chick pea leaves upto 2nd generation and the subsequent generations were reared by the method of Teakle and Jensen (1985) at 25 °C, 50 ± 10% relative humidity, with 14 h photoperiod using artificial diet. During oviposition, *H. armigera* adults were provided with 5% (w/v) honey solution.

“Quick screen” and toxicity assay against *Helicoverpa*

In order to ascertain the ability of native Bt isolates to demonstrate appreciable activity against second instar larvae (6 h after moulting) of *H. armigera*, “quick screen” bioassays were performed (Rampersad and Ammons 2005). “Surface contamination” method involving higher doses (1mg of total protein mL⁻¹ of culture supernatant) of the actively growing cultures (18 h) smeared onto young chick pea leaves in triplicate were employed. The treated larvae were left for 10 h then presented with a fresh piece (uninoculated) leaf and left further for 12 h. Scoring was done as; not eaten a score of 3; partially eaten a score of 2; completely eaten a score of 1. The average of three replicates was taken as toxicity value, which were arbitrarily classified as; 1.0- non-toxic; > 1.0 but < 2.0 – uncertain toxicity; ≥2.0 - toxic. All the putative *B. thuringiensis* isolates in the collection were tested. Bt subsp. *kurstaki* HD-1 was used as positive control and solubilisation buffer as negative control. The conditions for bioassay were as; 25 °C, 50 ± 10% RH and a 14:10 (light/dark [h]) photoperiod. Strains showing score of ≥2.0 were subjected to further bioassays to know their toxicity potential using four different concentrations (50, 100, 200 and 400 ng) of Vip protein cm⁻² of the leaf surface, 10 larvae per treatment were used and the experiment was set in triplicate making total number of larvae used per concentration to 30. Mortality was recorded at 3 days after treatment and the data was analysed using Probit Analysis (Finney 1971) to determine the LC50 (lethal concentration required to kill 50% of larvae tested). The amount of protein used in bioassays was determined according to Bradford (1976) and the partially purified and concentrated protein samples of toxic isolates were analysed by 10% SDS-PAGE gels.

Nucleotide accession numbers

Accession numbers of the partial 16S rDNA nucleotide sequences (KJ125306-KJ125391) and partial gene sequences of *vip1* (KJ396903-KJ396910), *vip2* (KJ396911-KJ396917), *vip3* (KJ396918-KJ396932) obtained in this study are available in NCBI GenBank database (<http://www.ncbi.nlm.nih.gov/genbank/submit.html>).

Results

Isolation and identification of *Bacillus thuringiensis* from soil

Out of the 86 *Bacillus* like isolates, 44 were identified as *B. thuringiensis* based on the presence of parasporal crystals and sequencing of 16S rRNA gene (Table S1). Different shapes of crystals (bipyramidal, spherical, irregular) were observed under Phase Contrast Microscopy and confirmed by SEM analysis of the crystalline inclusions (Fig. 1). The average Bt index observed in this study was 0.51 (Table 1).

Analysis of 16S rDNA sequences

To determine the phylogenetic relationship among the *Bacillus* isolates a 1500 bp amplicon corresponding to 16S rRNA was successfully amplified and sequenced in all the 86 presumptive *Bacillus* isolates. The partial sequences were aligned with the 16S rDNA sequences of Bt available from GenBank (CP004069, CP003763; CP000485, AB617500, JQ669397, CP001907, EU429670) and compared using Bioedit (Version 7.2.0). It was observed that the obtained sequences showed 95-100% identity with the reported sequences on BLAST analysis. The partial 16S rDNA sequences were submitted to GenBank and accession numbers were obtained as mentioned earlier. Of the 86 isolates tested, 80 showed maximum identity with *Bacillus sp*, only 6 isolates were identified as non-*Bacillus* viz. JK51, JK69, JK77, JK83, JK86 and JK95 which showed maximum identity with *Enterobacter asburiae*, *Brevibacterium frigoritolerans*, *Staphylococcus xylosus*, *Novosphingobium resinovorum*, *Brevibacterium frigoritolerans* and *Staphylococcus epidermidis* respectively

(Acc. Nos, KJ125355, KJ125365, KJ125373, KJ125378, KJ125381, KJ125390, respectively). Among the *Bacillus* isolates, *B. thuringiensis* was the most abundant (44) followed by *B. megaterium* (14), *B. aryabhattai* (13), *B. simplex* (2), *B. subtilis* (2), *Lysinibacillus sphaericus* (2), *B. flexus* (1), *B. methylotrophicus* (1), *B. pumilus* (1). A tree representing the phylogenetic relationship among the 16S rDNA gene was constructed using Neighbor-joining method (Fig. 2) by MEGA (version 6.0). Nucleotide sequences other than those resembling *B. thuringiensis* served as the remote control for phylogenetic analysis.

vip gene content of Bt in Kashmir valley

Forty four *B. thuringiensis* strains from Kashmir Valley were screened for the presence of *vip1*, *vip2* and *vip3* genes by using primer pairs Vip1smpF-Vip1smpR, Vip2smpF-Vip2smpR and Vip3AF-Vip3AR respectively (Table 2). Expected PCR amplicon sizes of 504 bp for *vip1*, 475 bp for *vip2* and 700 bp for *vip3A* type genes were observed (Fig. 3). *vip1* specific primers also amplified a non-specific fragment of <200 bp, which was not included in the study. The desired amplified products were purified and sequenced, the sequences on BLAST analysis showed identity to the corresponding reported sequences. The distribution of *vip*-type genes from different locations of Kashmir Valley was studied; *vip3* genes were found to be the most abundant (43.18%) followed by *vip1* (22.72%) and *vip2* (15.90%) genes. *vip3* gene specific sequences were abundant at Mairan (100%) Udusa (100%) and Gulmarg (57.14%). Forest soils of Kokernag and maize fields of Mairan and Udusa did not show the presence of either *vip1* or *vip2* sequences. All the 10 sites except Rajpora, showed presence of *vip3* gene sequences. *vip1* sequences were not found in *B. thuringiensis* isolates from any of the maize fields, whereas the abundance of *vip2* gene sequences was (16.66%) and (50.0%) in maize fields of Kalgi and Saripara respectively (Table 1). However, the presence of *vip3* sequences in the maize fields was the highest (50-100%). Isolates from three different soil types showed heterogeneous distribution of *vip* genes. Isolates from forest soils

harboured the maximum while as from lake sediment contained the minimum number of *vip* genes and profiles. *vip3A* gene was present in isolates from all three regions. However, the percentage of *vip3A* varied among three zones. The *vip1* and *vip2* genes were absent in isolates from lake sediments and maize fields respectively (Fig. 4). Though *vip1* and *vip2* are binary toxins, some Bt strains harboured either *vip1*-type genes or *vip2*-type genes. The *vip* gene sequences obtained from the Kashmir Valley in general were too conserved (showing more than 45% similarity to the existing *vip* sequences) to be assigned a new class, however the *vip1* and *vip2* sequences showed adequate differences (showing similarity of 64%) in amino acid sequence from the already reported sequences as analysed by protein BLAST and multiple sequence alignment (Fig. 5) compared to *vip3* gene sequences which were found to be highly conserved (showing similarity of 99% to the reported *vip3* sequences).

Protein purification

The PCR analysis (*vip3* gene sequence) and protein electrophoretogram (88 kDa protein) of the supernatant proteins strongly supported the presence of Vip3A proteins in selected isolates to study their insecticidal activity (Figs. 5 and 6a). Ammonium sulfate precipitation removed most of the contaminating proteins in the molecular weight range of interest (Fig. 6a). Heat treatment for inactivation of proteins in the supernatant was carried out at 95 °C for 20 min, insecticidal strains showed drastic (95-100%) reduction in mortality on heat treatment (data not shown). The active component was characterized as high molecular weight heat-labile component, thus excluding the possibility of being a β -exotoxin.

Toxicity assay

All the 44 putative *B. thuringiensis* isolates and the reference strain HD1 were subject to toxicity analysis. Leaves were partially eaten in majority (54.5%) of the cases and given a score of 1.0-2.0 (moderately toxic) followed by completely eaten leaves (29.5%), given a score of 1.0 (non- toxic), only seven isolates (15.90%) and a control strain achieved a score

greater than 2 and were considered as toxic (JK17, JK18, JK20, JK37, JK65, JK66, JK88 and HD1) (Fig. 6b). Bioassays of the putative toxic strains using culture supernatants containing different concentrations of Vip3A proteins revealed wide range of toxicity to *H. armigera*, with mortality ranging from 70% to 100%, mortalities were corrected using Abbott's (1925) formula. Among the native isolates JK37 followed by JK20 showed the highest mortality (96.60 % and 93.33 % respectively), and JK17 showed the least mortality (70.0 %), while as, Bt HD1 showed 100 % mortality when exposed to 400 ngcm⁻² of toxin. Though there was high magnitude difference in the LC50 values among the tested strains, all of them showed lower LC50 values compared to the reference strain HD1 (Table 3). Individual LC50s for *Helicoverpa* exposed to Vip3A toxins from different native isolates ranged from 115.968 ngcm⁻² to 246.605 ngcm⁻², while as, the HD1 strain showed the lowest LC50 value of 105.759 ngcm⁻² against *Helicoverpa*. The statistical results showed that the insecticidal activity of JK37 and JK88 are close to that of the standard strain HD1 (Table 3).

Discussion

Compared to Cry proteins that have been characterized extensively and used widely in insect management for last five decades (Federici 2005), studies on the diversity and distribution of Vip proteins is still in infancy. Although, distribution of *vip* genes of Bt in some geographical regions of India has been investigated earlier (Sattar and Maiti 2011; Shingote et al. 2013), no researcher had systematically analysed the distribution of *vip* genes in Kashmir valley. So it is of interest to determine the distribution and diversity of *vip* genes and identify the type of *vip* genes. In this study, Bt strains were characterized, the distribution of Bt strains and the type of *vip* gene in different soil types and their toxicity potential against lepidopteran pest *H. armigera* were examined.

Bt index is the measure of success in Bt isolation. The average Bt index in the present study was 0.51; it was 0.33 and 0.6 for soils from maize fields and forests respectively. Bravo et al.

(1998) reported Bt index of about 0.24 in cultivated fields of Mexico, Martin and Travers (1989) reported 0.85 Bt index in soils from various locations in Asia, Baig et al. (2010) reported 0.86 relative index from cattle waste compared to 0.69 from wheat grain dust from various habitats in different areas of Pakistan. 61.36% of isolates were obtained from forest soils, 25% from maize fields and 13.63% from lake sediment. Similar trend of Bt index was also observed in soils of Sichuan Basin in China (Yu et al. 2011).

Analysis of 16S rRNA sequences is a frequently used method for the identification and taxonomic localization of bacterial genus/species (Punina et al. 2013). However, early studies performed on isolates from *B. cereus* group revealed that the 16S rRNA sequences of Bt isolates in this group had as high as a 99–100% identity. Our findings also indicated the same trend showing identity as high as 95–100%. Since, *B. cereus* and *B. thuringiensis* species are indistinguishable from each other on the basis 16S rDNA sequence analysis (Punina et al. 2013), sequences showing maximum identity with either of the two have been considered as *B. thuringiensis* for construction of phylogenetic tree in this study. The topology of the constructed tree was in accordance with the phylogenetic structure of the *B. cereus* group as established by the analysis of the 16S rRNA, 23S rRNA gene fragments (Bourque et al. 1995) and the 16S–23S rRNA intergenic region (Daffonchio et al. 2000). Based on the phylogenetic analysis 16S rRNA sequences of all the native *B. thuringiensis* isolates grouped together along with the reference sequences of *B. thuringiensis* (Fig. 2), suggesting phylogenetic homogeneity among the *B. thuringiensis* strains, the same patterns were observed by Joung and Cote (2002).

PCR-based is the most widely used molecular approach for the identification of novel *cry* genes due to its rapidity and reproducibility and its use has also been extended to screening and identification of novel *vip* genes (Rang et al. 2005; Sattar and Maiti 2011; Shingote et al. 2013). Vip proteins account for about 15% of Bt strains has been reported by Wu et al.

(2007). In the present study, we found that the average rates of *vip1* (22.72%) and *vip2* (15.90%) were higher as compared to the rates described by other researchers (Yu et al. 2011; Shingote et al. 2013), all of whom described proportions of 10% for both *vip1* and *vip2*. The higher prevalence of *vip3* sequences in the present study to that of *vip1* and *vip2* sequences is corroborated with other studies (Beard et al. 2008; Palma et al. 2013). The difference in distribution of *vip1*, *vip2* and *vip3* can be ascribed to the difference in environmental conditions of these sites like altitude, nutritional conditions of the soil, oxygen concentration, temperature etc. (Yu et al. 2011; Shingote et al. 2013). *vip1* and *vip2* sequences obtained in the study were found to be variant from the reported *vip1* and *vip2* sequences based on the deduced amino acid sequence homology. Variation in a single amino acid has been proven to influence the level of toxicity in Vip and Cry proteins (Li et al. 2007; Ozturk et al. 2008), therefore the obtained sequences are a great reservoir to study their efficacy against Coleopteran insect pests. The variability in these gene sequences increases the possibility of developing broad spectrum Vip proteins, as proven earlier in case of chimeric Vip3AcAa (Fang et al. 2007). Therefore, potential of isolation and characterization of novel *vip* genes with improved toxicity and enhanced spectrum does exist in isolates under study. The analysis of *vip* gene sequences (Fig. 5) obtained revealed that different subclasses of *vip1* and *vip2* gene sequences were amplified using the degenerate primers designed in the study, thereby proving the robustness of the primers to amplify and differentiate among different subclasses of each family.

Vip3A is a group of proteins of approx. 89 kDa, known to be secreted by certain Bt strains during vegetative growth. It differs from Cry proteins both in amino acid sequence and mode of action and has been proven to be toxic to a wide spectrum of lepidopteran pests (Estruch et al. 1996; Selvapandiyan et al. 2001). We did not restrict toxicity studies against *Helicoverpa* to promising strains inferred by PCR analysis only, but to the entire collection of Bt strains

(44 isolates), second instar larval stage was used for bioassay, as older larvae tend to be more tolerant to Bt based biopesticides (Sanahuja et al. 2011). Only 15.9% of the Bt isolates displayed appreciable toxicity against *Helicoverpa* on “quick screen” bioassay analysis (Fig. 6b), these isolates were subjected to detailed toxicity analysis using different concentrations of the toxin.

In order to rule out the possibility of toxicity being the combinational effect of Vip3A with other proteins, heat treatment for inactivation of proteins in the supernatant was performed, which caused the reduction in mortality of the insecticidal strains as a result of Vip3A inactivation, thereby proving that the main active components of the supernatant are thermolabile Vip3A and not the thermostable compounds, β -exotoxin and zwittermicin A as proved earlier also (Estruch et al. 1996).

Despite the high level of *vip3A* conservation observed on PCR analysis, isolates harbouring the gene demonstrated a high level of heterogeneity with respect to their toxicity (LC50) against *Helicoverpa* larvae. The LC50 values of the native isolates ranged from 115.968 ng cm⁻² to 246.605 ng cm⁻², while as HD1 showed the lowest LC50 of 105.759 ng cm⁻². Earlier studies have also reported marked differences between the absolute values of LC50 for the Vip3A proteins; 325 ng cm⁻² by Doss et al. (2002), 155 ng cm⁻² by Liao et al. (2002), 251.98 ng cm⁻² by Shingote et al. (2013) against *H. armigera*. The likely reasons for the differences observed in LC50 may be due to one or more of the following factors: method of bioassay employed, insect diet composition, level of processing of toxins, the quantification method used, and the temperature at which bioassays were carried out.

To the authors' knowledge, this is the first systematic report on the diversity and distribution of *vip* gene sequences and the toxicity analysis against *Helicoverpa* in the locally isolated Bt strains of the valley. The study enriched the diversity of available *vip* genes, which thereby could broaden the spectrum of activity of Vip class of proteins and facilitate their application

for pest management (Estruch et al. 1997). We have developed a large and diverse *B. thuringiensis* strain collection with huge potential to control *Helicoverpa*; however, further research is needed to check their toxicity potential against other lepidopteran pests of agricultural importance. The 29.54% of the isolates that did not show amplification with any of the primers tested are of particular interest. Two *B. thuringiensis* strains JK37 and JK88 isolated in this study showed high toxicity towards *H. armigera*, moreover both the isolates showed the presence parasporal crystals on SEM analysis (Fig.1) indicating the presence of crystalline protein coding genes as well. These isolates have the potential to be developed as novel microbial biopesticides. Bioassay of the selected isolates against other lepidopteran pests, resistant insect pests and characterization of observed potentially novel *vip1* and *vip2* genes will be continued.

Acknowledgements

The first author (SAL) is grateful to the University grants commission (UGC), New Delhi for providing fellowship under Maulana Azad National Fellowship (MANF) scheme. Project Director, National Research Centre on Plant Biotechnology (NRCPB) is thanked for providing necessary facilities to carry out the research. National Agricultural Innovation Project (NAIP-C30026) of the Indian Council of Agricultural Research (ICAR), New Delhi is acknowledged for providing financial support. Scanning Electron Microscopy (SEM) facility provided by University Sophisticated Instrumentation Facility (USIF), Aligarh Muslim University (AMU), Aligarh is duly acknowledged. Dr. Daniel R. Zeigler, the *Bacillus* Genetic Stock Center (BGSC) Ohio State University, USA is thanked for providing the reference strain.

References

Abbott, W.S. 1925. A method of computing the effectiveness of an insecticides. J. Econ. Entomol. **18**: 265-267.

- 400 Altschul, S.F., Gish, W., and Miller, W. 1990. Basic local alignment search tool. *J. Mol. Biol.*
401 **215**: 403–410.
- 402 Baig, D.N., Bukhari, D.A., and Shakoori, A.R. 2010. Cry genes profiling and the toxicity of
403 isolates of *Bacillus thuringiensis* from soil samples against American bollworm, *Helicoverpa*
404 *armigera*. *J. Appl. Microbiol.* **109** (6): 1967–1978.
- 405 Beard, C.E., Court, L., Boets, A., Mourant, R.G., Van Rie, J., and Akhurst, R.J. 2008.
406 Unusually high frequency of genes encoding vegetative insecticidal proteins in an Australian
407 *Bacillus thuringiensis* collection. *Curr. Microbiol.* **57**: 195–199.
- 408 Bradford, M.M. 1976. A rapid sensitive method for the quantitation of microgram quantities
409 of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248-254.
- 410 Bravo, A., Likitvivatanavong, S., Gill, S.S., and Soberón, M. 2011. *Bacillus thuringiensis*: a
411 story of a successful bioinsecticide. *Insect Biochem. Mol. Biol.* **41**: 423–431.
- 412 Bravo, A., Sarabia, S., Lopez, L., Ontiveros, H., Abarca, C., Ortiz, A., Ortiz, M., Lina, L.,
413 Villalobos, F.J., Pena, G., Nuñez-Valdez, M., Soberon, M., and Quintero, R. 1998.
414 Characterization of *cry* Genes in a Mexican *Bacillus thuringiensis* Strain Collection. *Appl.*
415 *Environ. Microbiol.* **64** (12): 4965-4972.
- 416 Bourque, S.N., Valero, J.R., Lavoie, M.C., and Levesque, R.C. 1995. Comparative Analysis
417 of the 16S to 23S Ribosomal Intergenic Spacer Sequences of *Bacillus thuringiensis* Strains
418 and Subspecies and of Closely Related Species. *Appl. Environ. Microbiol.* **61**: 1623–1626.
- 419 Crickmore, N., Baum, J., Bravo, A., Lereclus, D., Narva, K., Sampson, K., Schnepf, E., Sun,
420 M., and Zeigler, D.R. 2014. *Bacillus thuringiensis* toxin nomenclature
421 <http://www.btnomenclature.info/>.
- 422 Daffonchio, D., Cherif, A., and Borin, S. 2000. Homoduplex and Heteroduplex
423 Polymorphisms of the Amplified Ribosomal 16S-23S Internal Transcribed Spacers Describe

- 424 Genetic Relationships in the “*Bacillus cereus* Group”. Appl. Environ. Microbiol. **66**: 5460–
425 5468.
- 426 Dhurua, S., and Gujar, G.T. 2011. Field-evolved resistance to Bt protein Cry1Ac in the pink
427 bollworm, *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) from India. Pest.
428 Manag. Sci. **67**: 898-903.
- 429 Estruch, J.J., Warren, G.W., Mullins, M.A., Nye, G.J., Craig, J.A., and Koziel, M.G. 1996.
430 Vip3A, a novel *Bacillus thuringiensis* vegetative insecticidal protein with a wide spectrum of
431 activities against lepidopteran insects. Proc. Natl. Acad. Sci. U.S.A. **93**: 5389–5394.
- 432 Estruch, J.J., Carozzi, N.B., Desai, N., Duck, N.B., Warren, G.W., and Koziel, M. 1997.
433 Transgenic plants: an emerging approach to pest control. Nat. Biotechnol. **15**: 137–141.
- 434 Fang, J., Xu, X., Wang, P., Zhao, J., Shelton, A.M., Cheng, J., Feng, M., and Shen, Z. 2007.
435 Characterization of chimeric *Bacillus thuringiensis* Vip3 toxins. Appl. Environ.
436 Microbiol. **73**: 956–961.
- 437 Federici, B.A. 2005. Insecticidal bacteria: an overwhelming success for invertebrate
438 pathology. J. Invertebr. Pathol. **89**: 30–38.
- 439 Finney, D.J. 1971. Probit Analysis. Cambridge University Press, London.
- 440 Gassmann, A.J., Petzold-Maxwell, J.L., Clifton, E.H., Dunbar, M.W., Hoffmann, A.M.,
441 Ingber, D.A., et al. 2014. Field-evolved resistance by western corn root worm to multiple
442 *Bacillus thuringiensis* toxins in transgenic maize. Proc. Natl. Acad. Sci. U.S.A. **111**: 5141–
443 5146.
- 444 Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis
445 program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. **41**: 95–98.
- 446 Joung, K.B., and Cote, J.C. 2002. A single phylogenetic analysis of *Bacillus thuringiensis*
447 strains and *Bacilli* species inferred from 16S rRNA gene restriction fragment length

- polymorphism is congruent with two independent phylogenetic analyses. *J. App. Microbiol.* **93**: 1075-1082.
- Jukes, T.H., and Cantor, C.R. 1969. Evolution of protein molecules. *In* Mammalian Protein Metabolism. *Edited by* H.N. Munro. Academic Press, New York, pp. 21-132.
- Li, C., Xu, N., Huang, X., Wang, W., Cheng, J., Wu, K., and Shen, Z. 2007. *Bacillus thuringiensis* Vip3 mutant proteins: Insecticidal activity and trypsin sensitivity. *Biocontrol Sci. Techn.* **17** (7): 699-708.
- Lone, S.A., Malik, A., and Padaria, J.C. 2014. Applications of *Bacillus thuringiensis* for prevention of environmental deterioration. *In* Environmental Deterioration and Human Health. *Edited by* A. Malik, E. Grohmann and R. Akhtar. Springer, Netherlands, pp. 73-95.
- Martin, P.A.W., and Travers, R.S. 1989. Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. *Appl. Environ. Microbiol.* **55**: 2437-2442.
- Masson, L., Moar, W.J., van Frankenhuyzen, K., Bosse, M., and Brousseau, R. 1992. Insecticidal properties of a crystal protein gene product isolated from *Bacillus thuringiensis* subsp. *kenyae*. *Appl. Environ. Microbiol.* **58**: 642-646.
- Nei, M., and Kumar, S. 2000. *Molecular evolution and phylogenetics*. Oxford University Press, New York.
- Ozturk, E., Acik, L., Ayvaz, A., Bozdogan, B., and Suludere, Z. 2008. Isolation and Characterization of native *Bacillus thuringiensis* strains from soil and testing the bioactivity of isolates against *Ephestia kuehniella* zeller (Lepidoptera: Pyralidae) larvae. *Turk. J. Biochem.* **33**: 202-208.
- Palma, L., Ruiz de Escudero, I., Maeztu, M., Caballero, P., and Muñoz, M. 2013. Screening of *vip* genes from a Spanish *Bacillus thuringiensis* collection and characterization of two Vip3 proteins highly toxic to five lepidopteran crop pests. *Biological Control* **66**: 141-149.

- 472 Punina, N.V., Zotov, V.S., Parkhomenko, A.L., Parkhomenko, T.U., and Topunov, A.F.
473 2013. Genetic diversity of *Bacillus thuringiensis* from Different Geo-Ecological Regions of
474 Ukraine by analyzing the *16S* rRNA and *gyrB* Genes and by AP-PCR and saAFLP. *Acta*
475 *naturae* **5(1)**: 90-100.
- 476 Rampersad, J., and Ammons, D. 2005. A *Bacillus thuringiensis* isolation method utilizing a
477 novel stain, low selection and high throughput produced atypical results. *BMC Microbiol.* **5**:
478 52. doi:10.1186/1471-2180-5-52.
- 479 Rang, C., Gil, P., Neisner, Van Rie, J., and Frutos, R. 2005. Novel Vip3-related protein
480 from *Bacillus thuringiensis*. *Appl. Environ. Microbiol.* **71**: 6276-6281.
- 481 Sampson, K., Dunn, E., Zeigler, J., and Tomso, D. 2009. Discovery of novel pesticidal
482 protein genes in *Bacillus thuringiensis* using *de novo* sequencing. *Annu. Meet. Soc.*
483 *Invertebrate Pathol.* 42nd, 2009. Abstr. B-8. p. 41.
- 484 Sanahuja, G., Banakar, R., Twyman, R.M., Capell, T., and Christou, P. 2011. *Bacillus*
485 *thuringiensis* a century of research, development and commercial applications. *Plant*
486 *Biotechnol. J.* **9**: 283–300.
- 487 Sattar, S., and Maiti, M.K. 2011. Molecular characterization of a novel vegetative insecticidal
488 protein from *Bacillus thuringiensis* effective against sap-sucking insect pest. *J. Microbiol.*
489 *Biotechnol.* **21**: 937–946.
- 490 Selvapandiyan, A., Arora, N., Rajagopal, R., Jalali, S.K., Venkatesan, T., Singh, S.P., and
491 Bhatnagar, R.K. 2011. Toxicity Analysis of N- and C-Terminus-Deleted Vegetative
492 Insecticidal Protein from *Bacillus thuringiensis*. *Appl. Environ. Microbiol.* **67(12)**: 5855-
493 5858.
- 494 Shingote, P.R., Moharil, M.P., Dhumale, D.R., Deshmukh, A.G., Jadhav, P.V., Dudhare,
495 M.S., and Satpute, N.S. 2013. Distribution of *vip* Genes, Protein Profiling and Determination

- 496 of Entomopathogenic Potential of Local Isolates of *Bacillus thuringiensis*. Bt. Research **4(3)**:
497 14-20.
- 498 Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. 2013. MEGA6: Molecular
499 Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution. Mol. Biol.
500 Evo. **30**: 2725-2729.
- 501 Teakle, R.E., and Jensen, J.M. 1985. *Heliothis punctiger*. In Handbook of insect rearing.
502 Edited by R. Singh and R.F. Moore. Elsevier, Amsterdam, pp. 312-322.
- 503 Travers, R.S., Martin, P.A.W., and Reichelderfer, C.F. 1987. Selective process for efficient
504 isolation of soil *Bacillus sp.* Appl. Environ. Microbiol. **53**: 1263-1266.
- 505 Van-Rensburg, J.B.J. 2007. First report of field resistance by stem borer, *Busseola fusca*
506 (Fuller) to Bt-transgenic maize. S. Afr. J. Plant Soil **24**: 147-151.
- 507 Vilas-Boas, G.T., and Lemos, M.V.F. 2004. Diversity of *cry* genes and genetic
508 characterization of *Bacillus thuringiensis* isolated from Brazil. Can. J. Microbiol. **50**: 605-
509 613.
- 510 Warren, G.W., Koziel, M.G., Mullins, M.A., Nye, G.J., Carr, B., Desai, N.M., Kostichka, K.,
511 Duck, N.B., and Estruch, J.J. 1996. Novel pesticidal proteins and strains. Patent
512 WO96/10083. World Intellectual Property Organization.
- 513 Wu, J.Y., Zhao, F.Q., Bai, J., Deng, G., Qin, S., and Bao, Q.Y. 2007. Evidence for positive
514 Darwinian selection of *vip* gene in *Bacillus thuringiensis*. J. Gene Genom. **34**: 649-660.
- 515 Yu, X., Zheng, A., Zhu, J., Wang, S., Wang, L., Deng, Q., Li, S., Liu, H., and Li, P. 2011.
516 Characterization of vegetative insecticidal protein *vip* Genes of *Bacillus thuringiensis* from
517 Sichuan Basin in China. Curr. Microbiol. **62(3)**: 752-757.

Table 1. Features of sampling sites, success of Bt isolation and the distribution of *vip* gene sequences.

Geographical Region (Kashmir Valley)	Soil Type	Total No. of colonies	No. of <i>Bacillus</i> like isolates ^a	No. of <i>Bt</i> isolates	<i>Bt</i> Index ^b	<i>vip</i> genes present		
						<i>vip</i> 1%/ no.	<i>vip</i> 2%/ no.	<i>vip</i> 3%/ no.
Brarinar 34° 6' N / 74° 20' E	Forest	186	18	12	0.66	8.33/1	33.33/4	25.00/3
Pangkong 33° 45' N / 78° 40' E	Lake sediment	18	8	6	0.75	16.66/1	0/0	33.33/2
Kalgi 34° 5' N / 74° 1' E	Maize field	154	11	6	0.54	0/0	16.66/1	50.00/3
Kokernag 33° 35' N / 75° 17' E	Forest	180	8	3	0.37	0/0	0/0	33.33/1
Mairan 34° 7' N / 74° 21' E	Maize field	40	7	1	0.14	0/0	0/0	100.0/1
Gulmarg 34° 3' N / 74° 23' E	Forest	25	7	7	1.00	85.71/6	14.28/1	57.14/4
Venkara 34° 14' N / 74° 20' E	Forest	102	6	5	0.83	40.00/2	0/0	40.00/2
Saripara 34° 16' N / 74° 23' E	Maize field	205	4	2	0.50	0/0	50.00/1	50.00/1
Rajpora 34° 9' N / 74° 23' E	Forest	20	6	0	0.00	0/0	0/0	0/0
Udusa 34° 5' N / 73° 54' E	Maize field	210	11	2	0.18	0/0	0/0	100.0/2
Total		1140	86	44	0.51	22.72	15.90	43.18

^a Off-white, opaque, slightly raised, and regular outlined.
^b Bt Index: *Bacillus thuringiensis* isolation index was calculated by dividing the number of Bt isolates by the total number of *Bacillus* like colonies obtained.

Table 2. Characteristics of partial gene primers used for identification of *vip* gene sequences in native *B. thuringiensis* isolates.

Primer Pair	Sequence (5' to 3') ^c	Respective gene	T _m (°C)	Amplicon size (bp)	Source
Vip1smpF	ATGAAGAAGRRSCTGRBSAG	<i>vip1</i>	55	504	This study
Vip1smpR	CTCCTTGAAGRTSTTGYTGTCsMTG				
Vip2smpF	CAAGGAGGACAAGGAGAAGGCC	<i>vip2</i>	55	475	This study
Vip2smpR	GSAYSAYGCCGGCCTTGGTGG				
Vip3AF	AGTTTACAAGAAATAAGTGTTA	<i>vip3A</i>	54	700	Selvapandiyan et al. (2001)
Vip3AR	CCTACCATTACATCGTGGAAT				

^c M = A + C; R = A + G; Y = C + T; B = C or G or T; S = C or G

Table 3. Insecticidal activity of the selected *B. thuringiensis* isolates against *H. armigera* (Lepidoptera: Noctuidae) larvae.

Strain tested	Slope ± SE	LC 50 (ng cm ⁻²)	95% Fiducial limits (ng cm ⁻²)	Toxicity index ^d
JK17	18.931±1.277	246.605	186.406-372.659	0.428
JK18	18.659±1.111	190.998	141.641-282.597	0.553
JK20	15.068±1.178	150.984	119.578-193.194	0.700
JK37	16.086±1.542	115.968	88.960-147.736	0.911
JK65	16.968±0.611	206.862	160.625-286.211	0.511
JK66	17.104±3.291	172.579	132.166-234.403	0.612
JK88	17.186±1.263	144.033	108.837-192.479	0.734
HD1	14.641±2.506	105.759	83.519-130.965	1.000

^dToxicity index indicates the relative toxicity of native to reference strain. It is the ratio of LC50 of HD1 to that of the tested strain; the larger the ratio, the higher the toxicity.

Fig. 1. Scanning Electron Microscopy of spore-crystals of *Bacillus thuringiensis* isolates showing different crystal shapes. Note: a-Isolates JK37, b-JK26, c-JK42, d-JK88; bc-bipyramidal crystal, hc-hexagonal crystal, sc-spherical crystal, ic-irregular crystal, sp-spore 169x107mm (300 x 300 DPI)

Fig. 2. A phylogenetic tree based on the 16S rDNA gene sequences of isolates belonging to the genus *Bacillus*, *Brevibacterium*, *Enterobacter*, *Lysinibacillus*, *Novosphingobium*, *Staphylococcus* obtained in this study was generated. Neighbor-joining method using MEGA version 6.0 was used for tree construction. The analysis involved 94 nucleotide sequences. Sequences from this study are prefixed with “JK” followed by strain number and GenBank sequence names are prefixed with accession numbers 182x371mm (300 x 300 DPI)

Fig. 3. PCR amplified products of different vip genes from representative Bt isolates. Lane M: 1 kb DNA Ladder 183x140mm (300 x 300 DPI)

Fig. 4. Distribution of vip genes in *Bacillus thuringiensis* isolates from various soil types identified by PCR Analysis 184x128mm (300 x 300 DPI)

Fig. 5. Amino acid sequence alignment of the partial Vip proteins, a) Vip1 and b) Vip2; sequence of the native isolates with the sequence of a representative of each subclass of Vip1 and Vip2 toxins. Light coloured (unshaded) areas indicate the divergent amino acids. The numbers indicates amino acid position 104x32mm (300 x 300 DPI)

Fig. 6. SDS-PAGE analysis and toxicity distribution of native *B. thuringiensis* isolates a) Protein profiles (10% SDS-PAGE gel) of culture supernatants of the putative toxic isolates (lanes 1-8) and partially purified Vip3A of the corresponding isolates (lanes 9-16); M indicates protein ladder. The 89 kDa-band indicated by arrow represents Vip3Aa protein; b) Distribution of *B. thuringiensis* collection based on their toxicity potential against *Helicoverpa armigera* larvae. The values indicate 1.0- not toxic; > 1.0 but < 2.0- moderate toxicity; = 2.0- toxic 198x178mm (300 x 300 DPI)

Fig. 1. Scanning Electron Microscopy of spore-crystals of *Bacillus thuringiensis* isolates showing different crystal shapes. Note: a-Isolates JK37, b-JK26, c-JK42, d-JK88; bc-bipyramidal crystal, hc-hexagonal crystal, sc-spherical crystal, ic-irregular crystal, sp-spore
169x107mm (300 x 300 DPI)

Fig. 2. A phylogenetic tree based on the 16S rDNA gene sequences of isolates belonging to the genus *Bacillus*, *Brevibacterium*, *Enterobacter*, *Lysinibacillus*, *Novosphingobium*, *Staphylococcus* obtained in this study was generated. Neighbor-joining method using MEGA version 6.0 was used for tree construction. The analysis involved 94 nucleotide sequences. Sequences from this study are prefixed with "JK" followed by strain number and GenBank sequence names are prefixed with accession numbers
 182x371mm (300 x 300 DPI)

Fig. 3. PCR amplified products of different vip genes from representative Bt isolates. Lane M: 1 kb DNA ladder
183x140mm (300 x 300 DPI)

Fig. 4. Distribution of *vip* genes in *Bacillus thuringiensis* isolates from various soil types identified by PCR analysis
184x128mm (300 x 300 DPI)

Fig. 5. Amino acid sequence alignment of the partial Vip proteins, a) Vip1 and b) Vip2; sequence of the native isolates with the sequence of a representative of each subclass of Vip1 and Vip2 toxins. Light coloured (unshaded) areas indicate the divergent amino acids. The numbers indicates amino acid position 104x32mm (300 x 300 DPI)

Fig. 6. SDS-PAGE analysis and toxicity distribution of native *B. thuringiensis* isolates a) Protein profiles (10% SDS-PAGE gel) of culture supernatants of the putative toxic isolates (lanes 1-8) and partially purified Vip3A of the corresponding isolates (lanes 9-16); M indicates protein ladder. The 89 kDa-band indicated by arrow represents Vip3Aa protein; b) Distribution of *B. thuringiensis* collection based on their toxicity potential against *Helicoverpa armigera* larvae. The values indicate 1.0- not toxic; > 1.0 but < 2.0- moderate toxicity; = 2.0- toxic

198x178mm (300 x 300 DPI)