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

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Abstract

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

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

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

Cry proteins have been extensively used for the control of various agriculturally important pests including the infamous cotton bollworm *Helicoverpa armigera*. However, there are several reports of field evolved resistance in *H. armigera* against Cry toxins throughout the 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 µg/mL. 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' AGAGTTTGATCCTGCTAG 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 µM each primer, 3 mM MgCl₂, 1.5 U of Taq DNA polymerase (Fermentas, USA) in a final volume of 25 µl. 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 mML⁻¹ NaCl containing 5 mML⁻¹ EDTA followed by resuspension in 0.02 M Tris, pH 8 and filtered (0.2 µm).

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 up to 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 \textit{vip} genes and profiles. \textit{vip}3A gene was present in isolates from all three regions. However, the percentage of \textit{vip}3A varied among three zones. The \textit{vip}1 and \textit{vip}2 genes were absent in isolates from lake sediments and maize fields respectively (Fig. 4). Though \textit{vip}1 and \textit{vip}2 are binary toxins, some Bt strains harboured either \textit{vip}1-type genes or \textit{vip}2-type genes. The \textit{vip} gene sequences obtained from the Kashmir Valley in general were too conserved (showing more than 45% similarity to the existing \textit{vip} sequences) to be assigned a new class, however the \textit{vip}1 and \textit{vip}2 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 \textit{vip}3 gene sequences which were found to be highly conserved (showing similarity of 99% to the reported \textit{vip}3 sequences).

**Protein purification**

The PCR analysis (\textit{vip}3 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 \textit{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 ng cm\(^{-2}\) 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 ng cm\(^{-2}\) to 246.605 ng cm\(^{-2}\), while as, the HD1 strain showed the lowest LC50 value of 105.759 ng cm\(^{-2}\) 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.
In the present study, we found that the average rates of \textit{vip}1 (22.72\%) and \textit{vip}2 (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 \textit{vip}1 and \textit{vip}2. The higher prevalence of \textit{vip}3 sequences in the present study to that of \textit{vip}1 and \textit{vip}2 sequences is corroborated with other studies (Beard et al. 2008; Palma et al. 2013). The difference in distribution of \textit{vip}1, \textit{vip}2 and \textit{vip}3 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). \textit{vip}1 and \textit{vip}2 sequences obtained in the study were found to be variant from the reported \textit{vip}1 and \textit{vip}2 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 \textit{vip} genes with improved toxicity and enhanced spectrum does exist in isolates under study. The analysis of \textit{vip} gene sequences (Fig. 5) obtained revealed that different subclasses of \textit{vip}1 and \textit{vip}2 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.

\textit{Vip}3A 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 \textit{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 *vip*3A 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.

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

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<tr>
<th>Geographical Region (Kashmir Valley)</th>
<th>Soil Type</th>
<th>Total No. of colonies</th>
<th>No. of Bacillus like isolates&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of Bt isolates</th>
<th>Bt Index&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>vip2%/ no.</td>
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<td>Rajpora</td>
<td>Forest</td>
<td>20</td>
<td>6</td>
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<td>0.00</td>
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<td>Udusa</td>
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<td>210</td>
<td>11</td>
<td>2</td>
<td>0.18</td>
<td>0/0</td>
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<sup>a</sup> Off-white, opaque, slightly raised, and regular outlined.

<sup>b</sup> 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.

<table>
<thead>
<tr>
<th>Primer Pair</th>
<th>Sequence (5’ to 3’)§</th>
<th>Respective gene</th>
<th>T&lt;sub&gt;m&lt;/sub&gt; (°C)</th>
<th>Amplicon size (bp)</th>
<th>Source</th>
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<tr>
<td>Vip1smpF</td>
<td>ATGAAGAAGRRSCTGRBSAG</td>
<td><em>vip</em>1</td>
<td>55</td>
<td>504</td>
<td>This study</td>
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<tr>
<td>Vip1smpR</td>
<td>CTCCTTAGRRTTTGTYTGTCMTG</td>
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<tr>
<td>Vip2smpF</td>
<td>CAAGGAGGACAGGGAAGGCC</td>
<td><em>vip</em>2</td>
<td>55</td>
<td>475</td>
<td>This study</td>
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<tr>
<td>Vip2smpR</td>
<td>GSAAYGCGCCCTGGTGG</td>
<td></td>
<td></td>
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<tr>
<td>Vip3AF</td>
<td>AGTTCAGAAATAAGTGTTA</td>
<td><em>vip</em>3A</td>
<td>54</td>
<td>700</td>
<td>Selvapandiyan et al. (2001)</td>
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<tr>
<td>Vip3AR</td>
<td>CCTACCATTACATCGTGAAT</td>
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</tbody>
</table>

§ 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.

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

\(^d\)Toxicity 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)
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169x107mm (300 x 300 DPI)
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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)
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