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Frequency distribution of lepidopteran-specific secretory genes in indigenous *Bacillus thuringiensis* isolates recovered from diverse soil habitats of India

Mamta Gupta, Sarvjeet Kaur and Ashika Debbarma

Abstract
Bacillus thuringiensis (*Bt*) is an ubiquitous entomopathogenic bacterium present in almost all environments and predominantly in soils. It produces secretory toxins during its vegetative growth phase, in addition to other virulent factors. Among these secretory proteins, the Vip3 proteins have been reported to be effective against major lepidopteran pests of several agricultural crops, thus spurring identification of *Bt* isolates which produce Vip proteins in different laboratories world over. Therefore, screening of *Bt* isolates recovered from various soil habitats in India was carried out in this study to analyze the effect of type of soil on the distribution of *vip3* genes. We have observed heterogeneous distribution (20%-100%) of *vip3*-type genes in *Bt* isolates, which may be due to ecological, topographical and nutritional conditions of soil. The overall frequency of occurrence of *vip3*-type genes was not significantly different in *Bt* isolates recovered from soil samples of cropped and non-cropped areas. However, isolates recovered from forest soil were the least abundant in *vip3*-type genes. We have found that our collection from various soil habitats is a rich source of *vip3*-type genes, which can be further explored for isolation of full length unique *vip3*-type genes having toxicity against agriculturally important lepidopteran insects for deployment in insect pest control.

Keywords: *Bacillus thuringiensis*, lepidopteran pests, *vip* genes, soil habitats

1. Introduction
Insect pests are the major challenging factor for augmenting crop yields in order to improve world agricultural economy. Insect pests belonging to the order Lepidoptera are among the most detrimental pests [1]. *Bacillus thuringiensis* (*Bt*) bacterium is found in various natural surroundings and plays a very significant role in the control of agriculturally important insects due to presence of crystalline, cytotoxic and secretory proteins, which have entomopathogenic properties [2]. The crystalline proteins have been used extensively in the formulated biopesticide sprays against various agricultural pests since past five decades. The *cry* toxins have also been successfully employed to develop insect pest-resistant transgenic crops since1996 [3]. Despite increasing commercial cultivation of *Bt* transgenic crops in several countries, continuous expression of *cry* genes in transgenic crops has led to emergence of resistant field population of insects, which is a major challenge for agricultural scientists. Furthemore, Cry proteins currently available in the global database are not effective to control a significant number of insects [4, 5, 6]. With this vision, there has been an urgent need to study genetic variability of this bacterium for identification of specific virulence factors other than crystalline proteins for long-term benefits.
In 1996, it was first reported that *Bt* strains secreted Vip (Vegetative insecticidal protein) proteins during vegetative growth phase, which do not share homology and receptor binding sites with Cry proteins [7, 8, 9, 10]. Contrasting features were found between Vip3 and Cry proteins with reference to onset of symptoms, which are postponed by 36-48 h after ingestion of Vip proteins as compared to that induced by crystalline toxins [11]. Vip3 proteins have been reported to be similarly effective as Cry proteins, and have different amino acid sequence and mechanism of action as compared with Cry proteins, which makes Vip proteins worthy candidates for deployment in gene pyramiding strategies to delay development of resistance in target insect pests [12, 8, 13]. The full potential of Vip3 proteins is relatively lesser known, despite the fact that there are 111 Vip3-type proteins identified and cloned till date [14]

(http://www.lifesci.sussex.ac.uk/home/NeilCrickmore/Bt/vip.html accessed on 10.05.2020). So far, the Vip3 proteins have been reported to be effective against black cut worm *Agrotis ipsilon* [11, 12], cotton boll worm *Helicoverpa armigera* [15, 4], beet army worm *Spodoptera exigua* [12, 15], fall army worm *Spodoptera frugiperda* [11, 16, 17, 18], tobacco bud worm *Heliothis virescens* [7, 19], tobacco horn worm *Manduca sexta* [8], African cotton leaf worm *Spodoptera littoralis*, European grapevine moth *Lobesia botrana*, cabbage moth *Mamestra brassicae* [20, 21], Mediterranean flour moth *(Ephestia kuehniella*) [22], cabbage looper *Trichoplusia ni* [4], tomato leaf miner *Tuta absoluta* [23], diamond back moth *Plutella xylostella* [24, 19, 20], locust bean moth *Ectomyelois ceratoniae* [25] and tobacco cut worm *Spodoptera litura* [24, 26].

A habitat's microbial network depends on the nature of its soil. However, despite *Bt* being a major inhabitant of soil, a detailed study of *vip* gene distribution in various kind of soils has not been undertaken. Since India is rich and unique in biodiversity and genetic assets, diverse Indian *Bt* isolates are a useful repertoire for identification of new types of toxic genes [27]. Therefore, in the present investigation, we have analyzed correlation between occurrence of *vip3* genes and the nature of soil from which particular isolates have been recovered, to determine the potentiality of *Bt* isolates for their activity against lepidopteran pests.

2. Material and Methods

2.1. Bacterial isolates

Bt isolates have been recovered from diverse habitats in India by Dr. Sarvjeet Kaur at ICAR-National Institute of Plant Biotechnology (NIPB), New Delhi. We have explored 115 *Bt* isolates recovered from various soil habitats from this *Bt* collection. The glycerol cultures of isolates have been maintained at -80°C.

2.2 Growth conditions

The glycerol cultures were used to obtain single colony of each *Bt* isolate by streaking on Luria Agar (LA) medium and penicillin (10µg/ml) antibiotic in petridishes. After overnight incubation at 28°C, Petri plates were observed for growth of single colonies and stored at 4°C, or taken for further study.

2.3 Extraction of plasmid DNA

The starter culture was prepared by inoculation of single colony in Luria Bertani (LB) media with penicillin (10µg/ml) and incubated for 3-4 h at 28°C, with shaking at 200 rpm in a shaker incubator (Kuhner, Germany). Secondary culture was prepared by inoculation of 1 ml starter culture into 10 ml LB medium and kept overnight in shaker incubator at 28°C, 200 rpm. Thereafter, an alkaline lysis miniprep method modified from Birnboim and Dolly (1979) for plasmid DNA extraction was followed [28]. Quality and quantity of plasmid DNA was ascertained by gel electrophoresis on 0.8% Agarose.

2.4 Amplification of secretory genes

The primer pair used in this study was taken from Mesrati *et al* 2005 [22] with following details: forward primer V1F (ATGAACAAGAATAATACTA, 19 bp); reverse primer V2R (TCTATTTGCAGACTTAGCGC, 20 bp); size of amplified product, 419 bp. PCR reaction volume of (25 µl) was prepared using plasmid DNA (25 ng/µl), 10X supplemented PCR buffer (2.5 µl), 2mM deoxy ribonucleotide triphosphate (dNTPs, 2.5 µl), 1 µM primer V1F (1.5µl),1 µM primer V2R (1.5µl), 1.0 U MBI, Fermentas *Taq* DNA polymerase (0.1 µl) and remaining sterile nuclease free water. These reactions were performed in Thermal Cycler (BioRad Laboratories, USA) following temperature profile as 94°C for 1 min (initial denaturation), followed by 30 cycles of denaturation (94°C for 1 min), annealing (42°C for 1 min) and extension (72°C for 1 min);and afterwards, final extension (72°C for 10 min). The amplified products were observed and compared with 1 kb molecular marker (MBI Fermentas, Germany) on 0.8% agarose gel, stained with ethidium bromide and observed on Gel documentation platform (Syngene, UK).

3. Results

3.1 Distribution of secretory genes in *Bt* isolates from soils of different locations

All 115 isolates were grown to obtain single colony on Luria agar plates with selection marker penicillin (10 µg/ml) (Figure 1), which was used for plasmid DNA extraction and PCR analysis. This investigation gives insight into frequency of occurrence for *vip3*-like genes in isolates of soil samples taken from various habitats of cropped (62.61%), non-cropped (28.70%) and forest (8.70%) areas. Identification through PCR (Polymerase chain reaction) technique revealed 73.61%, 78.79% and 20% *vip3* gene frequency in isolates of cropped area, non-cropped area and forest area, respectively (Table 1, Figure 2). The expected *vip3* gene amplified product of 419 bp is depicted in Figure 3.

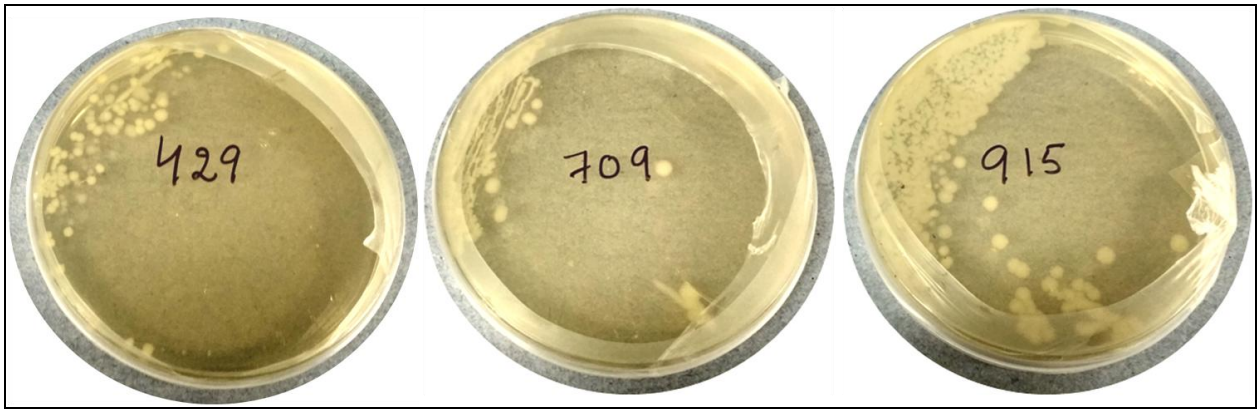


Fig 1: Representative picture of Luria Agar Petri plates with single colonies of each *Bt* isolate

Table 1: Frequency distribution of *vip3*-genes in *Bt* isolates recovered from cropped, non-cropped and forest areas

Site	*Bt* isolates (Number)	PCR-Positive isolates*	Percent *vip3*-frequency
Cropped Area	72	53	73.61%
Non-Cropped Area	33	26	78.79%
Forest Area	10	2	20%

*Presence of product of 419 size in PCR amplification

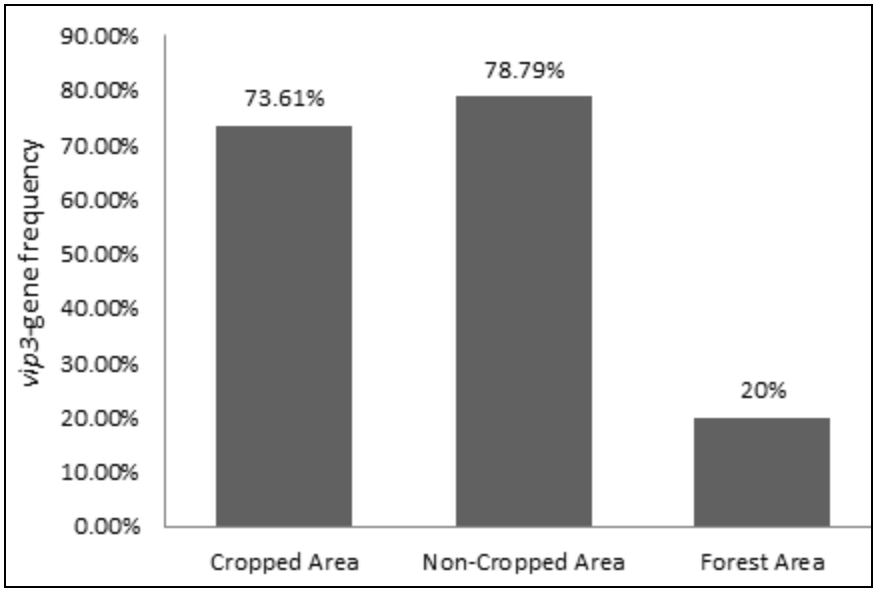


Fig 2: Frequency distribution of *vip3* genes in *Bt* isolates recovered from soil samples of diverse areas

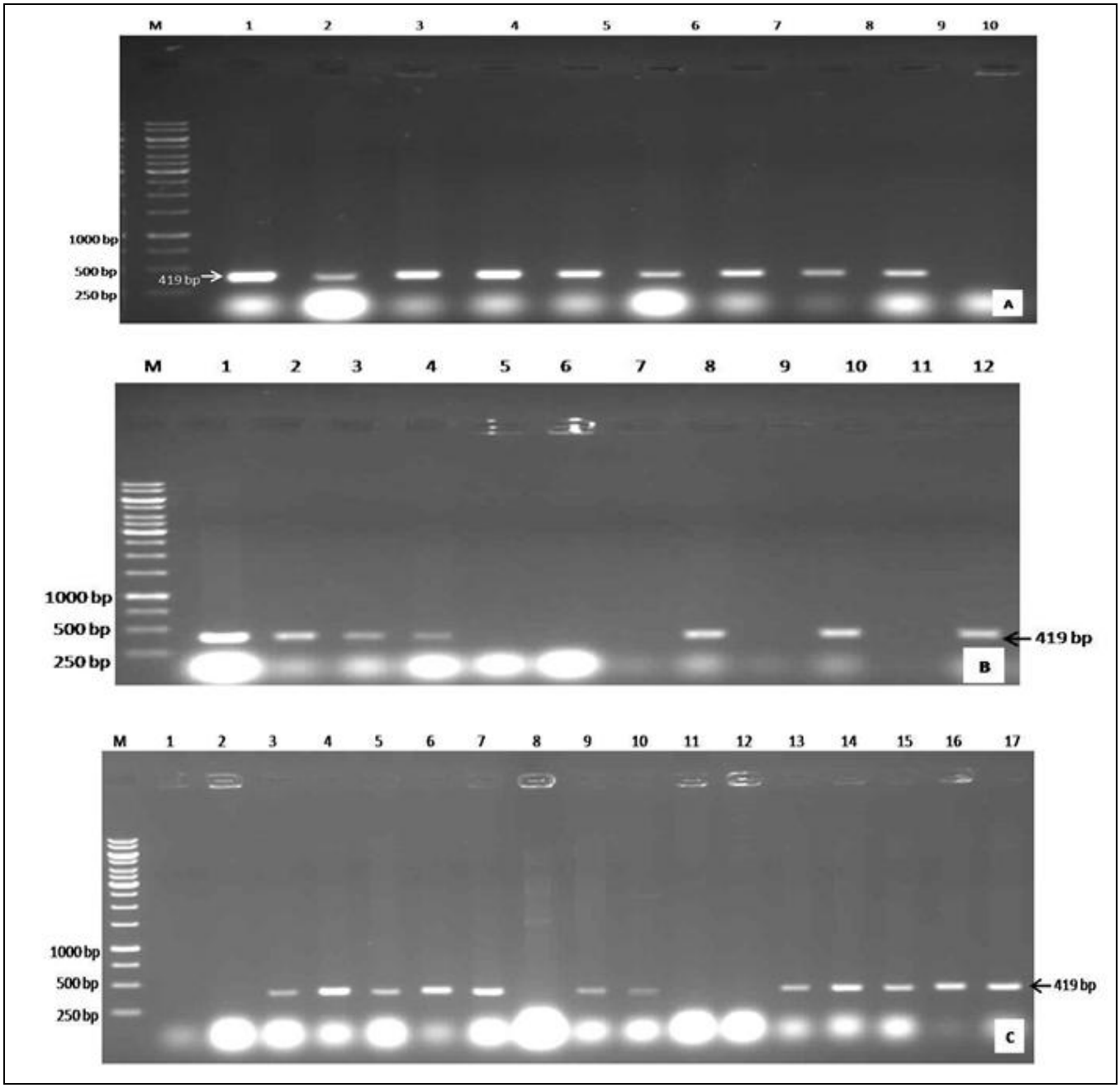
3.2 Distribution of secretory genes in isolates recovered from diverse soil habitats

This examination revealed that distribution of these secretory genes among total 115 soil isolates was varied, ranging from 20% to100% with respect to each soil habitat. Further detailed analysis revealed that maximum, 100% frequency was found in isolates recovered from soil of wheat, maize and paddy fields, cattle shed and Sangam River. This was followed by isolates from chilli field (85.71%), desert (80.95%), chickpea field (80%), infested field (75%), cotton field (72.41%), Orchard (66.67%), non-cultivated land (66.67%), kitchen garden (62.5%), fallow land (50%), red gram field (20%) and forest area (20%) soils (Table 2, Figure 4). This data showed that *vip3* genes are generally abundant in isolates belonging to soil from field crops. However, only 20% isolates recovered from soil samples from red gram field and animal sheds were observed to be positive for the presence of *vip3* genes.

Table 2: Frequency distribution of *vip3*-genes across isolates from various soil habitats

Site	Soil Habitat	*Bt* isolates (Number)	PCR-Positive isolates*	Percent *vip3*-gene frequency
Cropped Areas	Chick Pea Field	5	4	80
	Cotton Field	29	21	72.41
	Chilli Field	7	6	85.71
	Red Gram Field	5	1	20
	Wheat Field	6	6	100
	Maize Field	3	3	100
	Paddy Field	2	2	100
	Orchard Soil	3	2	66.67
	Infested Field	4	3	75
	Kitchen Garden Soil	8	5	62.50
Non-Cropped Areas	Cattle Shed	2	2	100
	Sangam River Soil	2	2	100
	Fallow Land	2	1	50
	Desert Soil	21	17	80.95
	Non -Cultivated Land	6	4	66.67
Forest Area		10	2	20

*Presence of product of 419 size in PCR amplification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1000 bp
500 bp
419 bp
250 bp
A
M 1 2 3 4 5 6 7 8 9 10 11 12
1000 bp
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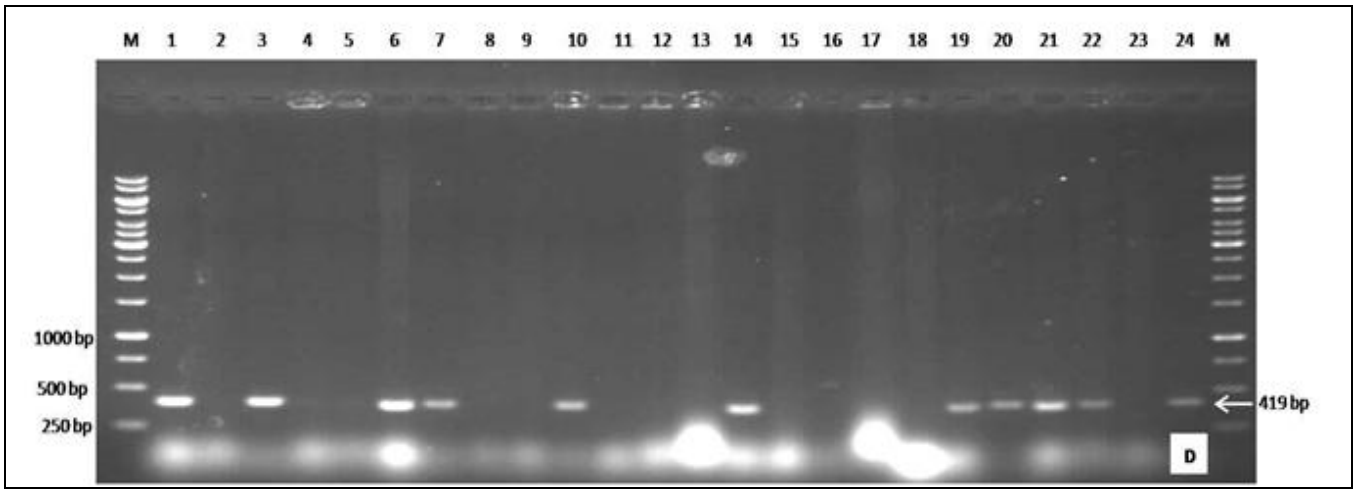
250 bp
419 bp
B
M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
1000 bp
500 bp
250 bp
419 bp
C


M 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 M
1000 bp
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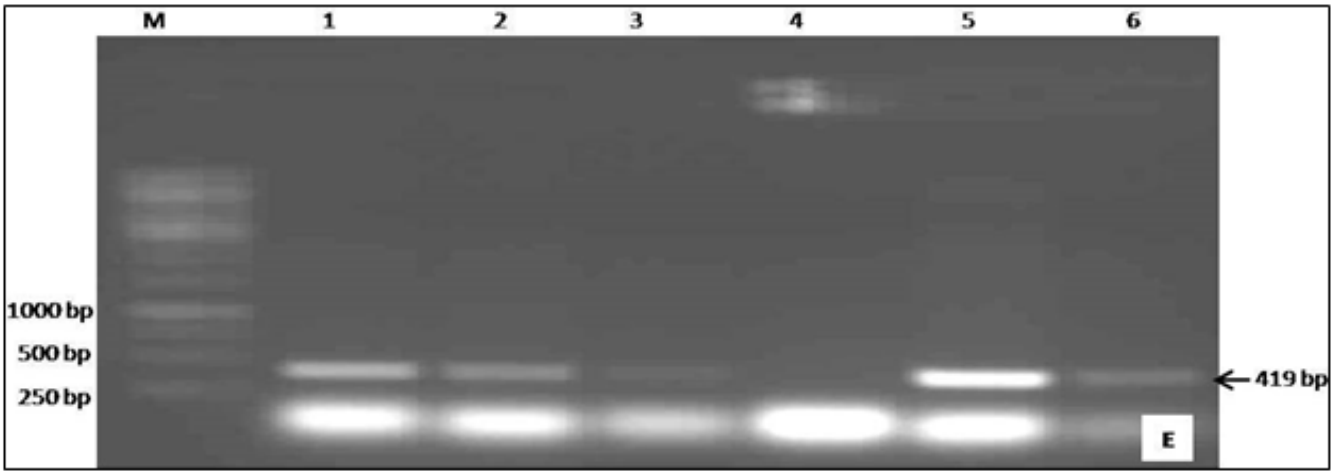

Fig 3: PCR analysis representing amplified product of 419 bp long *vip3* genes in indigenous *Bt* isolates recovered from diverse soil habitats A) M: 1kb DNA Ladder; 1: *Bt* reference strain HD1 used as positive control; 2 -4: Chickpea soil isolates; 5-7: Cotton soil isolate; 8-10: Orchard soil isolates B) M: 1kb DNA Ladder; 1: *Bt* reference strain HD1 used as positive control; 2 -3: Sangam river isolates; 4: Cotton soil isolate; 5-9: Red gram soil isolates; 10-12: Cotton soil isolates C) M: 1kb DNA Ladder; 1-2: Red gram soil isolates; 3-6: Wheat soil isolate; 7-8: Fallow land isolates; 9-10: Cattle shed isolates; 11-17: Desert soil isolates D) M: 1kb DNA Ladder; 1-3: Cotton soil isolates; 4-7: Non-cultivated land soil isolate; 8-15: Forest soil isolates; 16-19: Kitchen Garden soil isolates; 20-21: Paddy soil isolates; 22-24: Infested soil isolates; M: 1kb DNA Ladder E) M: 1kb DNA Ladder; 1: *Bt* reference strain HD1 used as positive control; 1-5: Chilli filed soil isolates

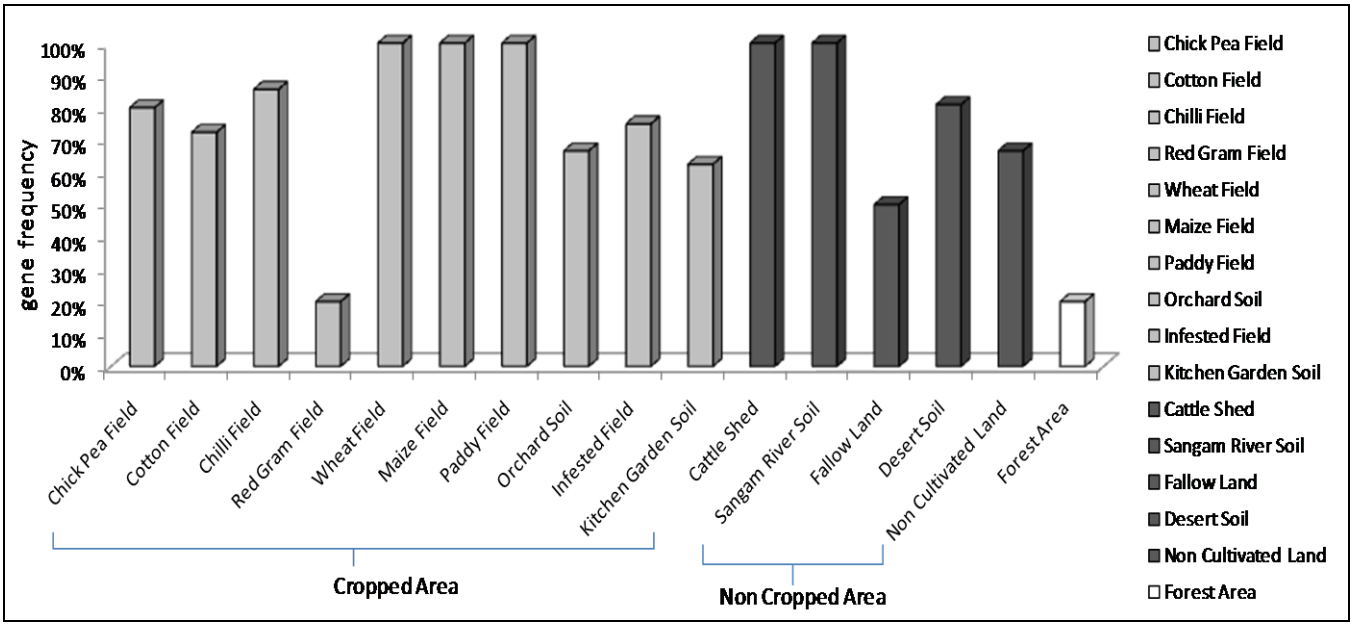

Fig 4: Frequency distribution of *vip3* genes in isolates from various soil habitats

Discussion

Bt microbe is extensively distributed in diverse ecosystems, irrespective of geographical area and habitats [29, 30]. It has been observed that by and large, this microbe exhibits genetic diversity with reference to different kinds of toxic groups, due to exchange of plasmids between its strains [31]. Distribution and variability of *cry* genes has been linked with diversity in the sampling sites, with respect to ecological, geographical and biological characteristics. Indeed, every natural surrounding has distinct *Bt* isolates, which may be expected to contain new toxic groups, having greater potential against target insects of different orders. Consequently, various *Bt* strains from diverse ecologies have been collected world over and characterized to assess their toxic effect on different insect orders [32, 33, 34, 35]. Thus, it is essential to recover numerous *Bt* isolates from diverse environments, for discovery of unique *B*t isolates having novel toxins with better and specific toxicity. It provides us the opportunity for biological control of devastating agricultural insect pests and also deals with the evolution of resistant insect pest populations. Among various strategies, PCR has been demonstrated to be a valuable technique for strain characterization with respect to toxic genes. The efficiency of PCR is based upon design of appropriate primers corresponding to presence of conserved regions in genes of interest. This approach is fairly sensitive, requires limited quantity of DNA and permits rapid and concurrent screening of numerous isolates, in order to anticipate their insecticidal properties [36, 37]. Although, a large number of *vip3* gene-related screenings have been reported with soil isolates of various countries, a systematic analysis regarding categorization and frequency distribution of *vip3* genes based on diverse soil habitats has not been done by many researchers.

Our results have shown prevalence of *vip3* genes in *Bt* isolates recovered from samples collected from a range of soil habitats

of different geographical regions. The presence of *vip3* genes in isolates from non-cropped area was higher than in isolates belonging to cropped area, but that frequency was not significantly different and similar range 20-100% was observed in isolates from both areas. Similar data has been observed for distribution of *cry* genes in isolates of agricultural and non-agricultural areas [38, 39]. This may be due to the ubiquitous nature of *Bt*, such that there was no significant difference in the presence of *vip3* genes among isolates from cropped and non-cropped area. Nevertheless, this study might be helpful to gain insight into distribution of *vip* genes in many soil habitats.

In a previous study, *Bt* isolates were recovered from a range of soil *viz*. red, purple, black, gray, alpine meadow, cinnamon, etc. taken from farmland, mountain, plains and forest regions of China and 8.4% -100% frequency of *vip3* genes was reported [40]. Screening of 146 isolates from soil, leaf litter, fresh foliage and other kind of substrates of different forest regions in Costa Rica was done and 54% were found to be positive for the presence of *vip3* genes. Among 146 isolates, 75 were recovered from forest soil samples and 41 (54.67%) isolates were detected with the presence of*vip3* genes [41]. Another study [42] reported 25%-100% frequency of *vip3* genes in isolates obtained from soil samples of forest, lake sediment and maize field of Srinagar, India. These authors have reported 50-100% and 25-57.4% frequency for maize field and forest area, respectively, which is in agreement with our observation for both areas and also corroborate the observations of Arrieta and Espinoza (2006) in respect to forest area. Likewise, distribution study of *cry1*, *cry2*, *cry3* and *cry4* gene reported prevalence of only *cry1* gene (63.3%) in isolates from forest soil of Ladakh, India [38]. However, contrary to our results, that is, 100% frequency for isolates from Sangam river, only 33.33% isolates from lake sediment were observed to be positive by Lone *et al*. 2016 [42]. Another study [43] did not find any isolate recovered from soil of vegetable and forest area in Assam with positive *vip3* gene. They found maximum positive isolates from paddy soil and minimum from tea garden soil. Ours and previous investigations have indicated that isolates belonging to cereal crop soils are rich in *vip3* gene abundance, whereas 20-55% frequency was observed in isolates from forest area soil.

We observed that within soil isolates from cropped area, relatively less frequency of *vip3* genes was in isolates of red gram soil (20%) whereas higher frequencies of 80% and 72.14% were observed in isolates of pulse crop chickpea and cash crop cotton soil, respectively. In this study, medium range frequency was found in isolates of orchard (66.67%) and kitchen garden (62.50%) with no considerable difference. However, 85.71% of isolates from chilli field were found to contain *vip3* gene, which was nearly in agreement with 97.3-100% frequency observed in isolates from cinnamon soil, [40] which represented the frequency in spices fields. This difference in abundance between kitchen garden and chili field may be due to different microclimate in kitchen garden, which is affected by surroundings of different kinds of vegetable crops as compared with chilli cultivated singly in one field.

In Rajasthan, seven (87.5%) desert soil *Bt* isolates out of 8, were observed to have *vip3A* gene [44] which was similar with our observation as 17 (80.95%) of 21 isolates from desert soil have been detected positive in our study. Therefore, isolates of desert soil are having abundance of *vip*3 genes, which maybe further explored for variation and potentiality. To the best of our knowledge, there are not many studies regarding distribution of respective genes in desert area soil.

This diversification of frequency distribution in various soil habitats maybe due to variation in ecological conditions of respective locations or habitats *viz*. soil atmosphere like amount of oxygen, its temperature and nutritional environment, its elevation, *etc*. [40, 45].

Conclusion

Present investigation describes the variation in the presence of *vip3*-type genes in isolates of samples taken from diverse kinds of soil environments in India and suggests that their occurrence is correlated with microenvironment and nutritional conditions existing in a particular soil habitat. We found heterogeneous distribution of *vip3* gene in different kind of soil types, which also confirmed the previous findings. PCR method greatly enhanced the efficiency of *vip3* gene detection in our collection, which has enabled us to identify isolates which can be used for full length gene amplification with specific primers in the near future. Additional experiments related to cloning and characterization of these genes, in order to gain information on their novelty based on sequence variation and to identify *Bt* isolates which produce unique *vip3* genes having significant toxicity potential against specific pest as compared to *cry* genes, will facilitate their use in insect pest management strategies.

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