Ann Microbiol (2017) 67:143–155
DOI 10.1007/s13213-016-1244-0

ORIGINAL ARTICLE

Genetic diversity and functional characterization of endophytic *Bacillus thuringiensis* isolates from the North Western Indian Himalayas

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Received: 4 August 2016 / Accepted: 25 November 2016 / Published online: 15 December 2016

Abstract A total of 15 endophytic *Bacillus thuringiensis* isolates were obtained from root nodules of six legumes (soybean, ricebean, gahat, frenchbean, lentil and pea). All of these isolates were characterized by the presence of one of two different types of crystalline inclusions (spherical and bipyramidal) and tolerance to a wide pH range (4–10; optimum 7.0) and NaCl concentrations up to 8%. Genetic diversity among the *B. thuringiensis* isolates was determined by repetitive extragenic palindromic PCR assays (rep-PCR) using the *Bacillus cereus*-repetitive extragenic palindromic, BOX, enterobacterial repetitive intergenic consensus sequence and $(GTG)_5$ primers. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis proteogram of the *B. thuringiensis* isolates revealed the presence of two major polypeptides (24.4 and 131.0 kDa). Maximum crystal protein profile was observed in the *B. thuringiensis* isolates producing the spherical crystal, while those isolates producing the bipyramidal crystal protein showed four four major polypeptides (24.4, 33.8, 81.2 and 131.0 kDa). The purified crystal protein profile of the *B. thuringiensis* isolates revealed the presence of only one major protein of 130 kDa mass. Isolates VRB1 and VLG15 possessing the *cry1* and *cry2* family genes demonstrated 100% mortality against first-instar larvae of the Bihar hairy caterpillar (lepidopteran pest). Our study of the ecological and molecular diversity among newly identified *B. thuringiensis* isolates suggests that these could be useful in planning new strategies for integrated pest management in sustainable agricultural systems.

Keywords *Bacillus thuringiensis* · Endophyte · Legumes · Genetic diversity · Crystal protein *cry* gene

Electronic supplementary material The online version of this article (doi:10.1007/s13213-016-1244-0) contains supplementary material, which is available to authorized users.

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Introduction

Interest in the exploration of microbial diversity has been spurred by the realization that microbes are essential for life since they perform numerous functions essential for proper functioning of the biosphere. The distribution and diversity of the ubiquitous bacterium *Bacillus thuringiensis* (*Bt*) include soil, silk farms, insects, stored crop products (Burges and Hurst 1977; Schnepf et al. 1998), phylloplane (Smith and Couche 1991; Bizzarri and Bishop 2008) and aquatic habitats (Iriarte et al. 2000). *Bacillus thuringiensis* is best known for its production of a proteinaceous parasporal inclusion [crystal (Cry) protein] during sporulation that is toxic to insect larvae (Aronson et al. 1986). These Cry proteins are coded by *cry* genes harbored in megaplasmids, although it has also been suggested that they are present in the chromosomes (Kronstad et al. 1983). *Bacillus thuringiensis* megaplasmids have been studied with the aim to locate *cry* genes or, more frequently, to characterize each strain, serotype, or any other sub-specific group (Arturo and Ibarra 2005).

Bacillus thuringiensis accounts for about 5–8% of the *Bacillus* spp. population in the environment. More than 130 species of lepidopteran, dipteran and coleopteran insects are infected by *B. thuringiensis*, and 68 serotypes (81 serovar/

varieties) of *B. thuringiensis* with a wide array of host range have been isolated and characterized (Dean 1984; Hastowo et al. 1992; Krattiger 1997). The success of *B. thuringiensis* can be attributed to the high efficacy of its insecticidal proteins and the existence of a diversity of proteins that are effective against a range of important pests. Another feature of *B. thuringiensis* is the presence of *cry* genes that encode for the insecticidal toxins required for the development of transgenic crop plants (Schnepf et al. 1998). Advances in the development of new *B. thuringiensis*-based biopesticides and the genetic manipulations of their genes for plant biotechnology depend on the variety of available strains with different insecticidal spectra and their corresponding *cry* genes (Wadman 1997; Allwin et al. 2007). Therefore, knowledge of the diversity and functionality of insecticidal *B. thuringiensis* is essential to our understanding of ecology, interaction with other organisms and development of broad-spectrum biocontrol agents. The enterobacterial repetitive intergenic consensus (ERIC) sequences, also known as intergenic repeat units are present in many copies in the genomes of *Escherichia coli*, *Salmonella typhimurium* and other enterobacteria (Hulton et al. 1991). In ERIC-PCR a band pattern is obtained by amplification of genomic DNA located between ERIC elements or between ERIC elements and other repetitive DNA sequences, with the generation of distinctive electrophoretic patterns among different strains. ERIC-PCR involves the use of primers of 22 nucleotides with high homology to repetitive intergenic sequences that are dispersed throughout the procaryotic kingdom (Versalovic et al. 1991; Valicente and Lana 2008). Thus, diversity available in nature has to be collected systematically, classified, analyzed and harnessed for beneficial applications.

The vast array of microbial activities and their importance to the biosphere and to human economies provide a strong rationale for understanding their diversity, conservation and exploitation. A wide variety of methods are used to distinguish the many *B. thuringiensis* strains, including analysis of biochemical and physiological characters, crystal morphology and protein pattern after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and molecular techniques.

The root nodules of leguminous plants are symbiotic organs induced by root nodule bacteria called rhizobia that are responsible for the fixation of atmospheric nitrogen and supply of fixed form of nitrogen to the plant system. In addition to rhizobia, some non-symbiotic bacteria have also been isolated from the root nodules of a wide range of legumes (Kan et al. 2007). These non-symbiotic bacteria have not been studied extensively compared to endophytes living in other tissues of plants. In order to understand the plant–bacteria interactions, it is essential to study the diversity of nodule endophytic bacteria and their impact and interaction with host plants.

Here we report the results of our investigation of endophytic *B. thuringiensis* present in root nodules of cultivated legumes in the North Western Indian Himalayas in terms of distribution, functional characterization and diversity. At the start of this study no information was available on the distribution and diversity of *B. thuringiensis* in root nodules of cultivated legumes in Indian Himalayas. Hence, our results on the endophytic *B. thuringiensis* isolates identified provide information on their occurrence within the plant tissue and interactions among the symbiotic bacteria, host plants and environmental factors. The primary aim of our study was to explore novel ecological niches and molecular diversity among endophytic *B. thuringiensis* isolated from root nodules of legumes.

Materials and methods

Isolation of endophytic *Bacillus thuringiensis* (*Bt*)

Root nodules of 25 different legume cultivars were obtained from the Experimental Research Farm (29°36′60″N, 79°37′58″; altitude 1250 m a.m.s.l.) of ICAR–Vivekananda Institute of Hill Agriculture, Hawalbagh, Almora, Uttarakhand located in the North Western Indian Himalayas. The nodules were collected from 45-to 50-day-old plants, and healthy nodules were selected for the isolation of endophytic *B. thuringiensis*. The nodules were first washed thoroughly, then surface sterilized with acidified $HgCl_2$ (0.1% $HgCl_2$ acidified with 1% HCl) for 2–3 min, followed by washing with sterile distilled water, treatment with H_2O_2 (5%) for 3 min and five washes with sterilized distilled water. The sterility of the nodules was reconfirmed by incubating the final surface wash water on a Luria–Bertani (LB) agar plate (Hi-Media Laboratories Pvt Ltd., Mumbai, India). Four nodules from each cultivar were macerated separately in a sterile centrifuge tube containing 1 ml LB broth (amended with 0.25 mM CH_3COONa), vortexed for 2 min and incubated at 28°C for 24 h, following which the tubes were subjected to a heat treatment at 80°C for 20 min in a shaking water bath (Travers et al. 1987). After the heat treatment, an aliquot (0.1 ml) from each tube was spread plated on Luria–Bertani agar and incubated for a further 48 h at 28°C. Those bacterial colonies which were heat stable were counted in each plate and examined under a phase contrast microscope for the presence of spores and crystals. Bacterial colonies producing crystal during sporulation were designated as *B. thuringiensis*. Fifteen distinct *B. thuringiensis* colonies were purified by repeated streaking on Luria-Bertani agar plates, then preserved in 60% glycerol stocks and stored at −80 °C. All experiments were conducted using fresh cultures.

Morphological, biochemical and physiological characterization

The isolates were characterized phenotypically to the genus level based on morphological, microscopic (Gram's staining),

cultural (oxygen requirement), biochemical (utilization of 35 different carbon sources and enzyme activity) and physiological (effect of temperature, pH and salt tolerance) characteristics using standard procedures (Collins and Lyne 1980; Holt et al. 1994). All media were obtained from Hi-Media Laboratories Pvt Ltd. All the experiments were carried out at 28°C.

DNA extraction from *B. thuringiensis*

Genomic DNA was extracted by inoculating 1 ml of bacterial suspension (10^7 cells ml^{-1}) of each isolate into 50 ml of LB broth in Erlenmeyer flasks (150 ml) and incubating the suspension for 18 h at 28 ± 1°C at a uniform shaking speed of 120 rpm. Cells were harvested by centrifugation at 8000 rpm for 20 min, washed with 0.85% normal saline and resuspended in 200 μl Tris EDTA Buffer (TEB). The cell pellet was lysed with a combination of 10 μl of SDS (10%) and 5 μL of proteinase K (10 mg ml^{-1}), followed by extraction with phenol:chloroform (1:1) and chloroform:isoamyl alcohol (24:1). The extracted DNA was dissolved in 20 μl TEB and used as the template for the PCR reactions (Harwood and Cutting 1990).

Molecular diversity analysis by repetitive extragenic palindrome sequence-based PCR analysis

Repetitive extragenic palindrome sequence-based PCR analysis (rep-PCR) is a DNA fingerprinting technique that is based on the presence of repeat sequences in the genome (Versalovic et al. 1994). rep-PCR fingerprinting has proven to be a simple, fast and reproducible method to analyze genetic diversity among strains of a great variety of organisms (De-Brujin 1992; Lupski and Weinstock 1992). Molecular diversity among *B. thuringiensis* isolates was determined usingrep-PCR with the following primers: (1) enterobacterial repetitive intergenic consensus (ERIC); (2) BOX sequence (BOX); (3) GTG_5 (GTG; De-Brujin 1992; Holt et al. 1994); (4) *Bacillus cereus*-repetitive extragenic palindromic (Bc-rep) sequence (Arturo and Ibarra 2005). PCR cycling was carried out in a reaction volume of 20 μl containing 50 ng of genomic DNA, 2 mM $MgCl_2$, 0.25 mM each deoxynucleotide triphosphate (dNTP), 0.25 μM of each primer and 1 U of *Taq* DNA Polymerase (Bangalore Genei Pvt Ltd., Bengaluru, India). A DNA engine programmable thermal cycler (Bio-Rad, Hercules, CA) was used for all amplification reactions [Electronic Supplementary Material (ESM) (Table 1a]. The amplified fragments were separated by horizontal electrophoresis on a 1.5% agarose gel (23 × 25 cm) supplemented with a final concentration of 5 μg ml^{-1} ethidium bromide, at 80 V for 18 h. The quick load DNA ladder III was used as a molecular marker. Gels were visualized under UV light and photographed with an Alpha Imager TM 1200 imaging system (UVP Inc., Upland, CA). Dendrogram cluster analyses were created using the Gel Compar II software package, version 3.5 (Applied Maths, Kortrijk, Belgium) with the unweighted pair grouping method with arithmetic mean algorithm and coefficient of cosine.

Cry gene profiling by PCR

The 15 root nodule isolates of *B. thuringiensis* were subjected to *cry* gene profiling by PCR using a variety of oligonucleotide primer pairs specific for the toxin-encoding genes present in the collection. The isolates were first characterized by using family primers (primers directed toward a specific gene class); based these initial results the isolates were further characterized with type-specific gene primers (primers used to identify a specific gene subclass or type). Previously described combinations of primers were used, as shown in ESM Table 1b. The PCR reaction mix consisted of 50 ng of total DNA, 1 mM reverse primer (-), 0.5 mM each forward primer, 3 mM $MgCl_2$, 200 nM dNTP and 2.5 U of *Taq* DNA polymerase in a final volume of 20 μl. All reactions were performed in a thermal cycler (Bio-Rad) with an initial 5-min denaturation step at 94°C, followed by 30 cycles of amplification consisting of a 1-min denaturation at 94°C, a 45-s annealing at 45–51 °C based on primer the combination (ESM Table 1b) and a 2-min of extension at 72°C, with a final extra extension step of 10 min at 72°C. The PCR products were then electrophoresed on a 1% agarose-EtBr gel and the amplified DNA bands visualized in a gel documentation system (Alpha Image Analyser; Alpha Innotech Corp. Santa Clara, CA).

Protein profiling of *B. thuringiensis* isolates

Crystal proteins of *B. thuringiensis* isolates were isolated using the method of Attathom et al. (1995) with minor modifications. The *B. thuringiensis* isolates were grown for 24 h on LB agar plates at 28°C and the cells harvested by scraping in 10-ml sterilized distilled water. The pellet was first washed three times with saline phosphate buffer (pH 7.0) and then with sterile distilled water before being re-suspended in 2 ml extraction buffer (2.5% SDS, 125 mM mercaptoethanol, 4 mM EDTA in 15 mM Tris-HCl, pH 8.8). Following the addition of 2 ml of alkaline solubilization buffer (50 mM Na_2CO_3 buffer, pH 10.0), the suspension was incubated at 37°C for 1 h. A 40-μl aliquot of 100 mM PMSF was then added to the solubilized protein suspension and the suspension centrifuged at 11,180 x *g* (Sigma model 2 K15, rotor No. 12132; Sigma-Aldrich, St. Louis, MO) at 4°C for 20 min and the supernatants collected. Proteins were precipitated by adding four volumes of chilled acetone for 16 h at –20°C, then centrifuged at 11,180 x g at 10°C for 15 min. The pellet was washed with 85% ethyl alcohol, air dried and dissolved in 200 μl extraction buffer that contains 1 mM PMSF and stored at −20°C until use. The protein concentration was determined in each sample (Bradford 1976). Protein samples (20 μg ml^{-1})

of *B. thuringiensis* isolates were resolved on 10% SDS-PAGE gels (20 h, 50 Vcm^{-1}) (Laemmli 1970). The protein bands were developed with Coomassie brilliant blue R250 stain and their molecular weight (kDa) was determined using analysis tools of Alpha Imager TM 1200 software.

Isolation of crystal protein and insect bioassay

The crystal protein was purified from the spore–crystal mixture by solubilizing the crystals in solubilization buffer (50 mM sodium carbonate buffer containing 10 mM dithiothreitol, pH-11). The isolate was grown on LB agar plates for 5 days to ensure complete autolysis of all cells. The bacterial growth was scraped and suspended in ice cold crystal wash solution I (0.5 M NaCl; 2% Triton X-100) for 10 min, following which the suspension was centrifuged at 7000 rpm for 10 min; the resulting pellet was washed twice with ice cold solution of 0.5 M NaCl and three times with sterile double distilled water. The spore–crystal mixture was then resuspended in 2–4 ml of solubilization buffer and incubated at 37°C for 3–4 h with slow shaking, after which it was centrifuged at 10,000 rpm for 15 min and the supernatant collected. The supernatant, which containied the solubilized crystal protein, was subjected to protein precipitation with four volumes of ice cold acetone for 1 h at –20°C. The precipitated crystal protein was dissolved in 15 mM Tris-HCl containing 4 mM EDTA (pH 8.8) and stored at −20°C until use. Protein toxin content was estimated by the Bradford method using bovine serum albumin as standard.

The insecticidal activity of the *B. thuringiensis* strains was established through bioassay studies on first-instar larvae of the Bihar hairy caterpillar, *Spilosoma obliqua*, by the leaf-disc method. Spore–crystal formulation of each isolate was prepared by the acetone–lactose co-precipitation method (Dulmage et al. 1970), and Cry protein was quantified by the Bradford assay. Prewashed (with 0.1% Triton X-100) soybean leaves were cut into 5-cm-diameter leaf discs with a metal punch, and 200 μl of 10 μg ml^{-1} toxin solution, prepared using the spore–crystal formulation of each isolate in distilled water, was evenly spread out on both sides of each disc. After drying for 1 h, each leaf disc was placed individually in a small petri plate containing a moistened filter paper at the bottom. Ten neonate larvae were released into each petri plate and allowed to feed for 72 h before mortality was checked. A total of 30 larvae were tested for each isolate, with a simultaneous control treatment with distilled water. All bioassays were carried out at room temperature of 28 ± 2°C, 60 ± 5% humidity and 14-h light/10-h dark photoperiod.

Data analysis

The PCR and protein profiles (banding patterns) were visualized as orange and blue bands, respectively, in the gel documentation system (Alpha Imager TM 1200), and the photographs were documented. The bands were scored as 1 for the presence and as 0 for the absence of a band. The data collected were normalized and subjected to diversity analysis using cluster analysis with the criteria of Jaccard distance index (Jaccard 1908), using NTSYS-pc version 1.80 software (Rohlf 1998.). For maximum accuracy of comparison, all isolates were processed with the same batch of PCR master mix.

Results

Isolation and distribution frequency of *B. thuringiensis* isolates

A total of 15 *B. thuringiensis* isolated were obtained from surface-sterilized root nodule samples of 16 *kharif* [soybean (*Glycine max*); ricebean (*Vigna umbellata*), gahat (*Macrotyloma uniflorum*) and frenchbean (*Phaseolus vulgaris*)] and 9 *rabi* [lentil (*Lens culinaris*) and pea (*Pisum sativum*)] legume cultivars (Table 1). The frequency of endophytic *B. thuringiensis* isolates in the 25 different leguminous cultivars was comparable (*Bt* index 0 to 21.4×10^{-4}) to that of total endophytic heat-resistant bacteria. The highest frequency of endophytic *B. thuringiensis* (*Bt* index 21.4×10^{-4}) was recorded in one of the lentil cultivars (VL4), while none of the endophytic *B. thuringiensis* was found in pea and frenchbean cultivars. The maximum number of isolates found in any single cultivar was four, in soybean cultivar (VLS64), followed three isolates in lentil cultivar VL4. In general, the distribution of endophytic *B. thuringiensis* was quite low in the nodules of the sampled leguminous cultivars.

Morphological, biochemical and physiological characteristics of endophytic *B. thuringiensis*

All isolates, with the exception of VLS64.3 had a colony morphology that was irregular in shape with an undulate margin and wrinkled surface. All isolates were Gram positive and rod shaped and produced spores and crystal at the terminal position (Table 2). Based on the morphology of the crystals produced by the 15 isolates of *B. thuringiensis*, the isolates were divided into two groups, namely, those which produced spherical crystals and those which produce bipyramidal crystals (Fig. 2a). The highest frequency of bipyramidal and spherical crystal protein production was 53.3 and 46.7%, respectively, with larger bipyramidal crystals produced by VRB1 isolates.

All *B. thuringiensis* isolates were positive for starch hydrolysis, casein hydrolysis, catalase, oxidase and the lecithinase test and negative for cellulase, chitinase, pectinase, phosphatase, DNase, H_2S production and the methyl red test (ESM Table 2). Isolates VL4b(i), VL4b (ii) and VLS64.2 and VLS64.3 were negative for urease and gelatinase. Isolate

Table 1 Distribution frequency of endophytic *Bacillus thuringiensis* isolates in root nodules collected from different leguminous crop cultivars

Isolate identification no.	Crop species/Cultivars	Total heat-resistant colonies (CFU)	Confirmed *B. thuringiensis* isolates (n)	*Bt* index[a] ($\times 10^{-4}$)
A	Soybean (*Glycine max*)			
1	VLS72	6.0×10^3	3	5.00
2	VLS64	7.5×10^3	4	5.33
3	VLS63	1.3×10^4	0	0.0
4	VLSTc-1	3.0×10^3	0	0.0
5	VLS21	1.1×10^4	1	0.95
6	VLS2	3.5×10^3	0	0.0
7	VLS47	4.5×10^3	0	0.0
B	Ricebean (*Vigna umbellata*)			
1	VRB1	8.5×10^3	1	1.18
2	VRB53	1.2×10^3	0	0.0
3	VRBIC-176563	9.5×10^2	0	0.0
C	Frenchbean (*Phaseolus vulgaris*)			
1	VL Bouni Bean-1	2.5×10^2	0	0.0
D	Gahat (*Macrotyloma uniflorum*)			
1	VLG8	3.5×10^3	0	0.0
2	VLG10	1.1×10^4	0	0.0
3	VLG15	9.5×10^3	1	1.05
4	VLG19	5.5×10^3	0	0.0
5	VLG20	7.5×10^3	0	0.0
E	Lentil (*Lens culinaris*)			
1	VL1	3.5×10^3	0	0.0
2	VL2	9.0×10^3	1	1.11
3	VL3	5.5×10^3	0	0.0
4	VL4	1.4×10^3	3	21.42
5	VL126	1.7×10^3	1	5.88
6	VL507	7.0×10^3	0	0.0
F	Pea (*Pisum sativum*)			
1	VL42	1.6×10^3	0	0.0
2	VL45	8.0×10^3	0	0.0
3	VL47	6.0×10^3	0	0.0
Total	25 cultivars	1.2×10^5	15	1.23

CFU, Colony-forming units

[a] *Bt* index = Number of colonies of *B. thuringiensis* (CFU)/total number of bacterial colonies examined (heat-resistant bacteria, in CFU)

VLS72.3 was found negative for Vogues–Proskaur (VP) test. The carbohydrate utilization pattern showed that all the isolates were positive for dextrose, citrate and malonate and negative for lactose, galactose, raffinose, melibiose, sucrose, L-arabionose, inulin, sodium gluconate, glucasamine, dulcitol, inositol, sorbitol, manintol, adonitol, *C*-methyl-D-mannoside, ribose, cellobiose, melezitose, α-methyl-D-mannodide, xylitol, ONPG (lactose fermentation), esculin, D-arabinose and sorbose.

Physiological characterization of isolates included an assay of growth on a wide range of salt concentrations and pH. All isolates were found to have pH tolerance in the range of pH 5 to 9, while isolates VLS72.1, VLS72.3, VLS64.1 and VL126 were able to tolerate acidity to pH 4, and few isolates were also able to tolerate alkalinity to pH 10. However, optimum growth of most of the isolates occurred at pH 7–8. Similarly, the salt tolerance of all the isolates was in the range of 1 to 7% of NaCl, while isolate VL4b (ii) was able to grow on medium containing 8% NaCl (ESM Table 2).

Genetic diversity among *B. thuringiensis* using repetitive sequence-based PCR fingerprinting

The diversity among *B. thuringiensis* isolates was determined by rep-PCR, and a complex fingerprint pattern was obtained

Table 2 Isolation and morphological characteristics of endophytic *Bacillus thuringiensis* isolates

Isolate designation	Cultivar designation	Colony morphology[a]	Spore and crystal position	Crystal type[b]
VLS72.1	Soybean VLS72	IUWU*	Terminal	Spherical
VLS72.2	Soybean VLS72	IUWU*	Terminal	Spherical
VLS72.3	Soybean VLS72	IUWU*	Terminal	Spherical
VLS64.1	Soybean VLS64	IUWU*	Terminal	Spherical
VLS64.2	Soybean VLS64	IUWU*	Terminal	Spherical
VLS64.3	Soybean VLS64	ICSR	Terminal	Spherical
VLS64.4	Soybean VLS64	IUWF	Terminal	Spherical
VLS21	Soybean VLS21	IUWR	Terminal	Bipyramidal
VRB1	Rice bean VRB1	IUWF	Terminal	Bipyramidal
VLG15	Gahat VLG15	IUWU*	Terminal	Bipyramidal
VL4b(i)	Lentil VL4	IUWR	Terminal	Bipyramidal
VL4b(ii)	Lentil VL4	IUWR	Terminal	Bipyramidal
VL4C	Lentil VL4	IUWF	Terminal	Bipyramidal
VL2d	Lentil VL2	IUWF	Terminal	Bipyramidal
VL126	Lentil VL126	IUWR	Terminal	Bipyramidal

[a] I, Irregular; U, undulate; W, wrikled; U*, umbonate; C, curled; S, smooth; R, raised; F, flat

[b] Contains two types of crystal (larger and smaller); both are attached at the terminal end during sporulation

for all of the isolates studied with the BOX, ERIC, GTG and Bc-rep primers (Fig. 1a). The banding patterns of isolates from different legume cultivars were highly discriminative. The Bc-rep-PCR pattern of six *kharif* and two *rabi* isolates were similar, while the others had diverse patterns. The Box-PCR pattern of *kharif* isolates VLS64.1, VLS21 and VLS72.1 and of *rabi* isolates VL126, VL2d and VL4C showed more diverse patterns, while the others had an identical banding pattern. The *kharif* isolates VLS21 and VLG15 and *rabi* isolates VL4b(i), VL2d and VL4C showed higher diversity in their Eric-PCR pattern than did the other isolates. Additionally, the VRB1 isolate producing the largest bipyramidal crystal protein showed a Eric-PCR pattern that was distinctive from that of the other isolates.

The rep-PCR banding pattern data were subjected to dendrogram construction, revealing two main clusters at a 64.0% level of diversity (Fig. 1b), with 13 isolates forming the first major cluster and only two lentil endophytes (VL2d and VL4C) forming the second cluster. The highly diverse first cluster branched into two subclusters, which further gave rise to three distinct minor clusters. The first minor cluster included seven soybean endophytes and one lentil endophyte (VL126), the second cluster included only isolate VL4b(ii) and the third cluster included endophytes from soybean (VLS21), lentil (VL4b(i)), gahat (VLG15) and ricebean (VRB1). Two soybean isolates, VLS64.2 and VLS64.4, shared 96.0% genetic similarity and were diverse from the other soybean endophytes. Diversity among soybean endophytes ranged from 4.0 to 50.0%, with VLS21 being highly diverse from the other soybean isolates. Maximum diversity was observed in three *rabi* endophytes from the same lentil variety, namely, VL4C, VL4b(i) and VL4b(ii).

Protein fingerprinting of *B. thuringiensis* isolates

The SDS-PAGE patterns of *B. thuringiensis* isolates revealed the presence of four to eight polypeptides ranging in size from 20.4 to 131 kDa (Fig. 2a). Two major polypeptides of 24.4 and 131 kDa were common to all 15 *B. thuringiensis* isolates. The maximum protein profile (6–8 polypeptides) was observed in *B. thuringiensis* isolates produced a spherical crystal. All spherical crystal-producing isolates had the low molecular weight 20.3-kDa polypeptide, with the exception of the VLS72.1 and VLS64.2 isolates. Three spherical crystal-producing *B. thuringiensis* isolates, i.e. VLS72.2, VLS64.1 and VLS64.4, had 26.8-kDa crystal proteins. In comparison, isolates producing bipyramidal crystal proteins showed a lower protein banding pattern of four to five polypeptides ranging in size from 24.4 to 131 kDa. All bipyramidal crystal protein-producing *B. thuringiensis* isolates were found to have four major polypeptides, namely, 24.4, 33.8, 81.2 and 131.0 kDa. The 95 kDa polypeptide was present in all *B. thuringiensis* isolates producing bipyramidal crystal protein during sporulation, with the exception of VLS21, VRB1 and VLG15.

The dendrogram constructed using the SDS-PAGE proteogram revealed the presence of two major clusters among the isolates (Fig. 2b), with the first major cluster consisting of seven isolates producing spherical crystals and the second major cluster containing eight isolates producing bipyramidal crystal protein. The cluster composed of spherical crystal-producing isolates was highly diverse and branched into two

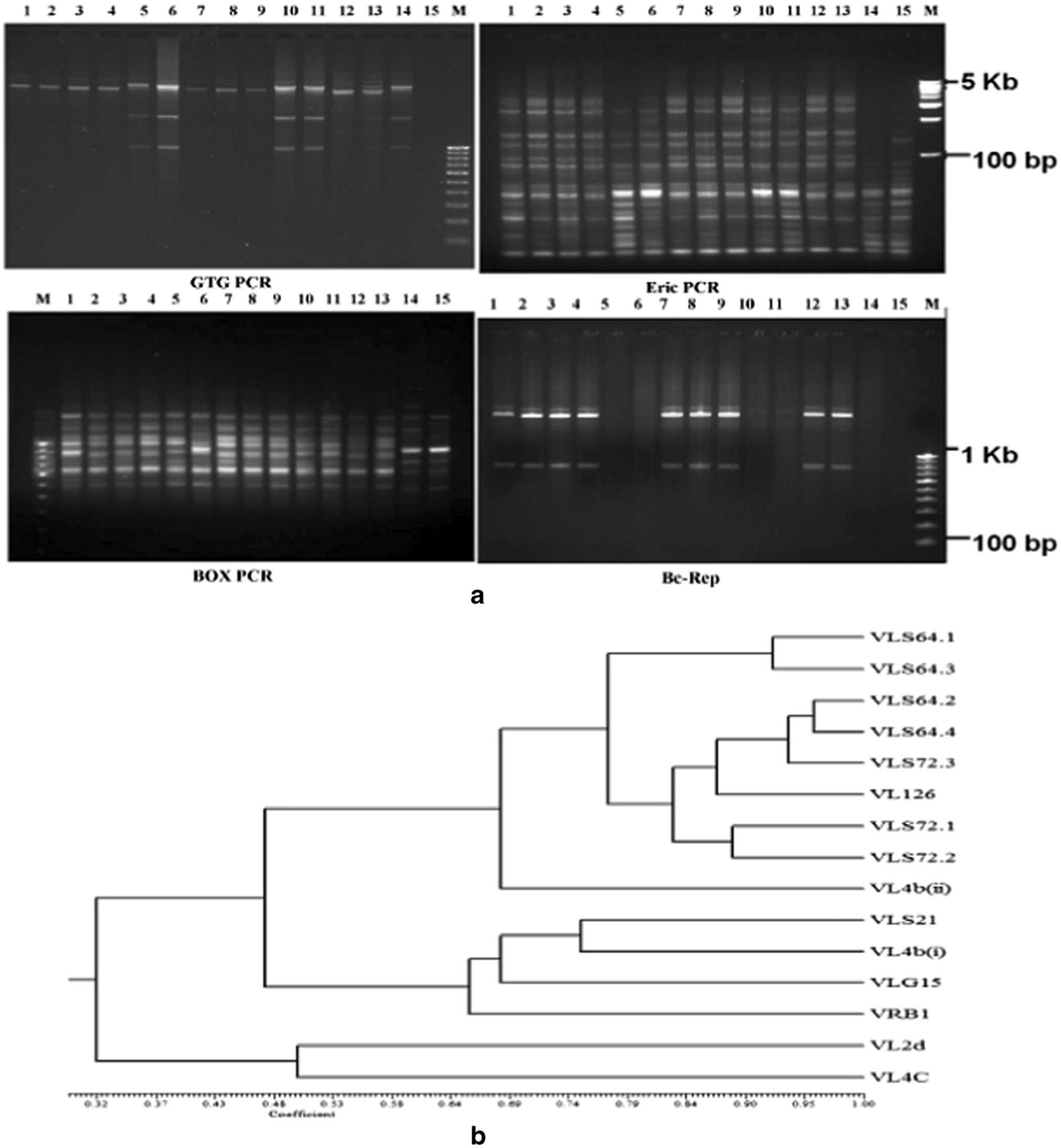

Fig. 1 **a** Representative PCR profiles following electrophoresis on 1.5% agarose gel and staining with ethidium bromide, showing polymorphism among the *Bacillus thuringiensis* isolates. DNA amplification was performed using was carried out using repetitive extragenic palindromic PCR (rep-PCR) with primers ERIC (enterobacterial repetitive intergenic consensus), BOX, GTG (GTG_5) and Bc-Rep (*Bacillus cereus*-repetitive extragenic palindromic. *Lanes M* Molecular weight marker (100 bp) ladder, *1–15 B. thuringiensis* isolates: *1* VLS64.1, *2* VLS64.2, *3* VLS64.3, *4* VLS64.4, *5* VLS21, *6* VLG15, *7* VLS72.1, *8* VLS72.2, *9* VLS72.3, *10* VRB1, *11* VL4b(i), *12* VL4b(ii), *13* VL126, *14* VL2d, *15* VL4C. **b** Unweighted pair grouping method with arithmetic mean dendrogram (UPGMA) resulting from rep-PCR patterns showing the genomic diversity among the 15 nodule endophytic *B. thuringiensis* isolates

subclusters, with first subcluster including six soybean endophytes and a separate branch for VLS64.2. Numerical analysis revealed that the two major clusters had the highest diversity coefficient of 0.35. The less diverse bipyramidal crystal-producing cluster branched into two subclusters, which is evident from the almost identical SDS-PAGE proteogram.

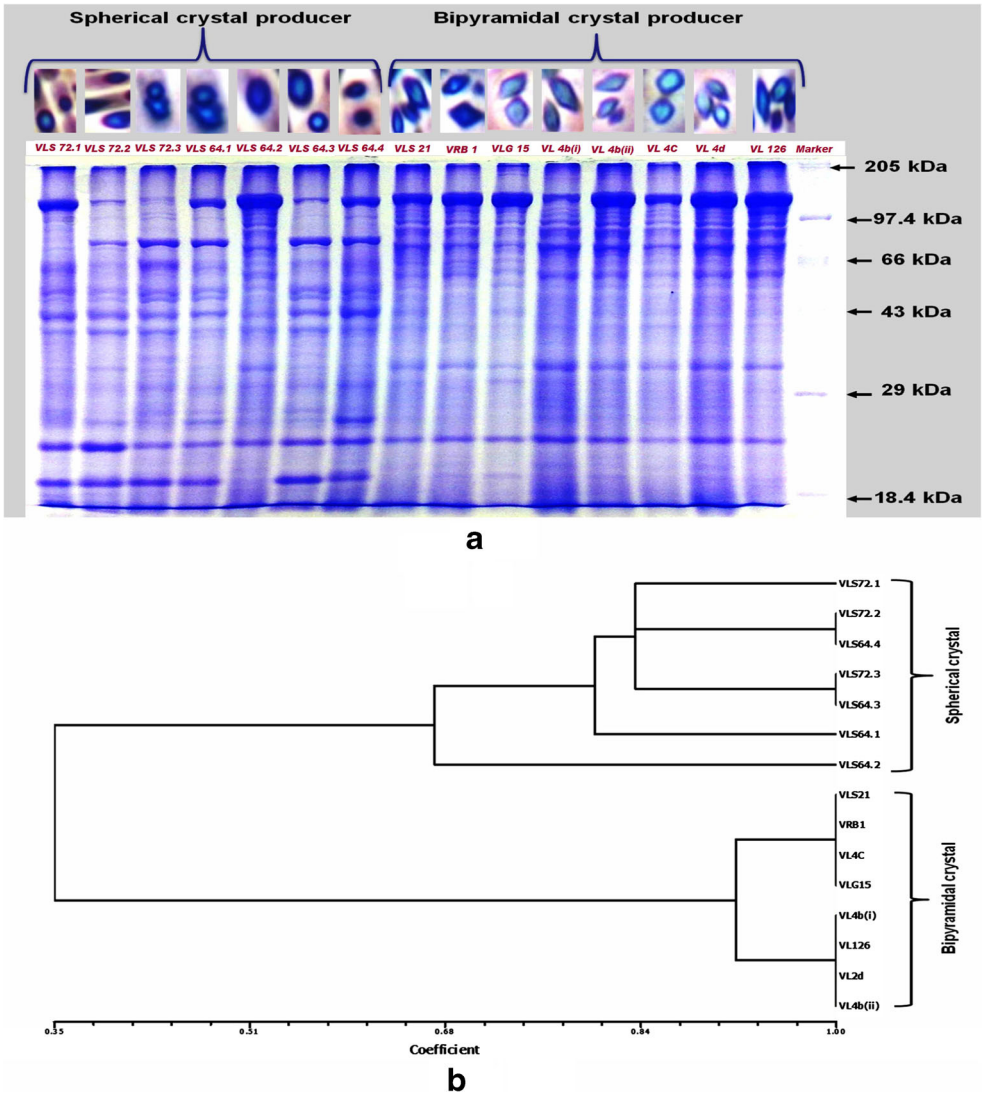

Fig. 2 **a** Morphology and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) profile of whole cell protein of endophytic *B. thuringiensis*. *Lanes 1–15 B. thuringiensis* isolates: *1* VLS72.1, *2* VLS72.2, *3* VLS72.3, *4* VLS 64.1, *5* VLS64.2, *6* VLS64.3, *7* VLS64.4, *8* VLS21, *9* VRB1, *10* VLG15, *11* VL4b(i), *12* VL4b(ii), *13* VL4C, *14* VL2d, *15* VL 126; *Marker* protein molecular weight marker. **b** UPGMA dendrogram resulting from SDS-PAGE profile of crystal protein of endophytic *B. thuringiensis* showing different clusters of different crystal composition

Diversity in *cry* genes and protein insecticidal activity

To determine the *cry* gene content of the 15 root nodule *B. thuringiensis* isolates we utilized a PCR technique specific for a known class of *cry* genes. The results showed the presence of a combination of *cry1* (*cry1Ab*, *cry1Ac*, *cry1C*, *cyr1D*) and *cry2* genes in nine isolates; the remaining six isolates were found to have only *cry* 8 genes (Fig. 3; Table 3). There was no evidence for the presence of the *cry1Aa*, *cry11B*, *cry2A*, *cry2B*, *cry3* and *vip3A* genes in any of the 15 isolates. With respect to the crops from which the *B. thuringiensis* strains were isolated, all lentil [VL4b(i), VL4b(ii), VL4C, VL2d and VL126], gahat (VLG 15) and ricebean (VRB1) isolates and two soybean (VLS21, VLS64.3) isolates were found to have a combination of the *cry1* and *cry2* family genes. Six *B. thuringiensis* isolates (VLS72.1, VLS72.2, VLS72.3, VLS64.1, VLS64.2 and VLS64.4) from soyabean root nodules were positive for *cry* 8 genes, and these isolates had no toxicity against the Bihar hairy caterpillar. This result explains the specificity of *cry* genes as they are the candidate genes for coleopteran insects.

Analysis of the purified crystal protein profile revealed the presence of one major protein of 130 kDa in all *B. thuringiensis* isolates (Fig. 4). Among the isolates positive for the *cry1* and *cry2* family genes, VRB1 and VLG15 showed the maximum mortality against the Bihar hairy caterpillar (100%), followed by VL4B(ii) and VL4C (96.6%) and VL4b(i), VL2d and VL126 (86.6%), while isolates VLS21 and VLS64.3 obtained from soybean showed 76.6 and 46.6% mortality, respectively, against this insect.

Discussion

Plants maintain a complex ecosystem in which bacterial communities interact continuously and compete for nutrients and water in the tissues of the host. Knowledge of the diversity of endophytic bacteria is important for both ecological and

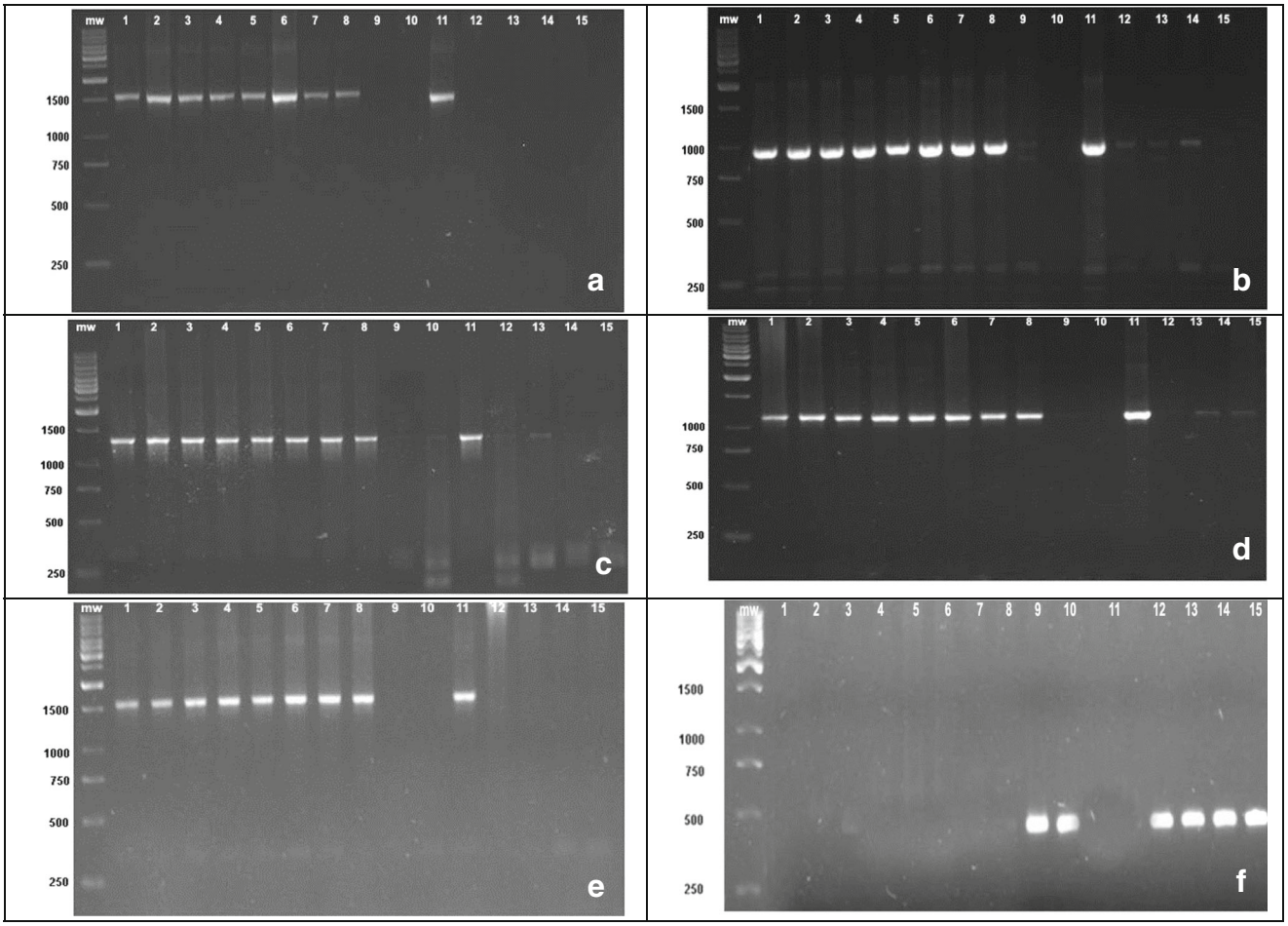

Fig. 3 Agarose gel (1%) electrophoresis of PCR products amplified from root nodule *B. thuringiensis* strains with the *cry1*-specific primers (**a**), *cry1Aa*-specific primers (**b**), *cry1Ac*-specific primers (**c**), *cry1C*-specific primers (**d**), *cry2*-specific primers (**e**), *cry8*-specific primers (**f**). *Lanes mw* Molecular weight marker (1 kb), *1–15 B. thuringiensis* isolates: *1* VL2d, *2* VL4c, *3* VL4bi, *4* VL4bii, *5* VL126, *6* VLG15, *7* VRB1, *8* VLS21, *9* VLS64.1, *10* VLS64.2, *11* VLS64.3, *12* VLS64.4, *13* VLS72.1, *14* VLS72.2, *15* VLS72.3

Table 3 Details of *cry* genes present and percentage mortality in Bihar hairy caterpillar (*Spilosoma oblique*) larvae caused by the 15 endophytic *B. thuringiensis* isolates

Bacillus thuringiensis isolates	*Cry* gene profile	Mortality (%) in *Spilosoma oblique*
VLS72.1	*Cry8*	0.0
VLS72.2	*Cry8*	13.3
VLS72.3	*Cry8*	0.0
VLS64.1	*Cry8*	3.0
VLS64.2	*Cry8*	0.0
VLS64.3	*Cry1Ab*, *1Ac*, *1C*, *1D*, *Cry2*	46.6
VLS64.4	*Cry8*	0.0
VLS21	*Cry1Ab*, *1Ac*, *1C*, *1D*, *Cry2*	76.6
VRB1	*Cry1Ab*, *1Ac*, *1C*, *1D*, *Cry2*	100
VLG15	*Cry1Ab*, *1Ac*, *1C*, *1D*, *Cry2*	100
VL4b(i)	*Cry1Ab*, *1Ac*, *1C*, *1D*, *Cry2*	86.6
VL4b(ii)	*Cry1Ab*, *1Ac*, *1C*, *1D*, *Cry2*	96.6
VL4C	*Cry1Ab*, *1Ac*, *1C*, *1D*, *Cry2*	96.6
VL2d	*Cry1Ab*, *1Ac*, *1C*, *1D*, *Cry2*	86.6
VL126	*Cry1Ab*, *1Ac*, *1C*, *1D*, *Cry2*	86.6

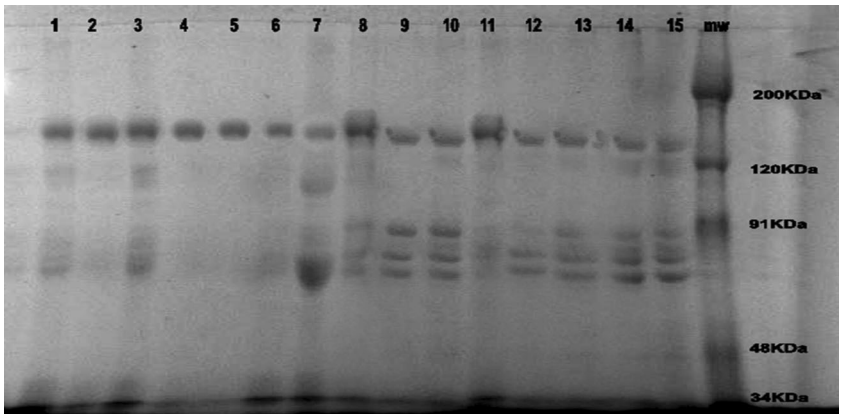

Fig. 4 SDS-PAGE studies of crystal proteins purified from root nodule inhabitant *Bacillus thuringiensis* isolates. *Lanes 1–15 B. thuringiensis* isolates: *1* VL2d, *2* VL4c, *3* VL4bi, *4* VL4bii, *5* VL126, *6* VLG15, *7* VRB1, *8* VLS21, *9* VLS64.1, *10* VLS64.2, *11* VLS64.3, *12* VLS64.4, *13* VLS72.1, *14* VLS72.2, *15* VLS72.3; *mw* high range protein molecular weight marker

biotechnological studies. The importance of *B. thuringiensis* as a biopesticide is now well recognized, and studies on *B. thuringiensis* crops have shown considerable promise for the promotion of *B. thuringiensis* as a genetic tool to create transgenic crops (Beatrix 1997). The potential environmental benefits of this approach have led to a reduction of the use of agricultural chemicals, and it is a strategy which fits well with integrated pest management modules. However, it is essential to continuously add to the genetic pool through a selection process of the newer *B. thuringiensis* strains to keep in check the pests that co-evolve with biological control agents.

In the present study, we collected root nodules from 45- to 50-day-old plants, and all of the sampled nodules were healthy based on their reddish color and hard texture. We excluded the possibility of endophytic bacteria taking advantage of the decay of nodules and soil contaminants since the nodules were surface sterilized, as evidenced by the absence of colony-forming units in the final wash water of the nodule surface sterilization process. The different *B. thuringiensis* isolates were confirmed on the basis of methods described by Travers et al. (1987), namely shape, size, Gram staining and the presence of spore crystals. All of the isolates were rod shaped, Gram positive and spore forming, and they produced crystal protein during sporulation. The high frequency of *B. thuringiensis* recovered from soybean and lentil root nodules indicated that *B. thuringiensis* predominantly occupy the nodules of these crop plants grown at the sampling sites, while they are not present in pea and frenchbean root nodules. This distribution frequency of the *B. thuringiensis* isolates indicates a specific interaction between *B. thuringiensis* and the leguminous host plant. Bai et al. (2002) and Zhang et al. (2012) also reported the isolation of putative endophytic plant growth-promoting, spore-forming bacteria from soybean root/nodules.

The 15 endophytic *B. thuringiensis* isolates were characterized based on polyphasic characterization (morphological, physiological, biochemical and genetic level). In practice, differentiation of *Bacillus* from other genera rarely presents any difficulty. The relatedness among the isolates identified in our study indicates the diversity of *B. thuringiensis* in different enzyme substrates and phenotypic characters, which is probably due to the ecological niche of the endophytic isolates. There was a disparate and diverse *B. thuringiensis* population within a single cultivar, as indicated by the fact that the distribution and frequency of *Bacillus thuringiensis* in a single cultivar differed from those in other cultivars. Our results show that *B. thuringiensis* isolates failed to grow anaerobically and that all isolates were positive for gelatin, casein hydrolysis catalase, citrate and the VP reaction, similar to the findings reported by Chatterjee et al. (2007) and Allwin et al. (2007).

The difference between endophytic *B. thuringiensis* isolates in the soybean, lentil, gahat and ricebean cultivars may be related to differences in plant genotypes, climatic conditions and soil conditions or soil microbial communities. However, other factors may also contribute the diversity between endophytic *B. thuringiensis* isolates of root nodule. One possible factor may be that legume seeds are sown in fields after they have been harvested and then stored by farmers for some time, allowing exposure to insects and birds and the build-up of *B. thuringiensis* from a wide variety of sources. *Bacillus thuringiensis* may also travel from location to location through air dust and by rainfall. In some cases, *B. thuringiensis* multiplies in cadavers of insects that have been killed by the *B. thuringiensis* toxin. These cadavers may themselves be ingested by lepidopteran larvae, scavenging insects, birds and mammals, and the spores spread in their feces (Burges and Jarrett 1990). Thus, there is likely to be a regular input of new strains (genotypes) of *B. thuringiensis* to the sampled location. Also, within the population of infected insects, there is the opportunity for transfer of the plasmid that codes for β-endotoxin genes from one bacterial strain to another (Jarrett and Stephenson 1990) by a conjugational-like process (Gonzalez et al. 1982). Thus, there is a potential for diverse populations of *B. thuringiensis* to emerge as a result of a natural plasmid transfer process in the environment, possibly affecting the distribution frequency of *B. thuringiensis* in root nodules of *kharif* and *rabi* legumes.

Our identification of a total of 98 non-symbiotic endophytic bacterial isolates indicated that almost every isolate had its own unique BOX-PCR pattern but one that was highly similar to those of endophytic bacteria isolated from the same sites (Li et al. 2008). We found that endophytic *B. thuringiensis* isolated from the same cultivar shared a high degree of identical BOX, ERIC, GTG and Bc-rep PCR patterns, indicating that they might be progeny of a single clone. This limited genetic diversity in the nodule endophytic *B. thuringiensis* isolates implies that the nodules might have a strong selection for the genomic background of endophytic bacteria in each cultivar, confirming that the plants specifically select endophytic bacterial genotypes (Siliciano et al. 2001). da Silva and Valicente (2013) also reported genetic diversity among *B. thuringiensis* using data obtained with ERIC, rep and BOX primers.

All *B. thuringiensis* isolates contain a set of self-replicating extrachromosomal DNA molecules or plasmids that vary in number and size in different strains (Andrup et al. 2003). Arturo and Ibarra (2008) used these plasmid patterns to characterize and compare 83 type strains of *B. thuringiensis* and 47 additional *B. thuringiensis* strains from six serotypes and found that some serotypes showed a great diversity of patterns, while other serotypes showed the same basic pattern among all its strains and a high degree of similarity. Such diversity may not be revealed by biochemical or serological tests (Martin et al. 1992) since these latter methods are not directly related to β-endotoxin genes, whereas the genetic and plasmid profile is a sensitive method for revealing the spectrum of genetic difference.

Grouping of *B. thuringiensis* isolates according to crystal protein profile studied by SDS-PAGE provided some data on the presence of diversity in *cry* genes. The *cry1Ab* gene encoded the 131-kDa Cry protein found in both spherical and bipyramidal crystalline inclusions that is active against lepidopterans. The 65-kDa Cry2 and 73-kDa Cry3 proteins are from bipyramidal and spherical crystalline inclusions, respectively. The cytolytic 26.57-kDa proteins encoded by the *cyt1A* gene and the 86-kDa protein from spherical inclusions are comparable to the CryV protein toxic to both coleopteran and lepidopterans (Dangar et al. 2010). The *Cry11b* gene encoding the 81.2-kDa proteins from bipyramidal crystalline inclusions is toxic to both coleopterans and lepidopterans (Ramalakshmi and Udayasuriyan 2010). Shishir et al. (2014) reported the isolation of *B. thuringiensis* strains from different ecosystems and their characterization based on biochemical typing, 16S rRNA gene analysis and plasmid and *cry* genes profiles. Based on these studies, it would appear that analysis of the crystal protein profile could be a useful tool to predict the presence of *cry* genes.

The search for novel *B. thuringiensis* strains may lead to the discovery of additional insecticidal proteins that are active against an emerging resistant insect population. Therefore, the larger the number of *B. thuringiensis* strains, the better our ability to facilitate the isolation of new types of *cry* genes. New variants of already known *cry* gene subgroups could encode crystal proteins with significant differences in the level and spectrum of toxicity due to variations in their sequences (Xue et al. 2008). Our observations demonstrate that in addition to proteins being either bipyramidal or spherical crystal, their composition was different and comparable to known toxins, supporting observations that they control a wide range of pests. Our analysis of these *B. thuringiensis* isolates showed that there were 56.2, 51.6, 42.8, 40.5, 33.8 and 20.3 kDa proteins, indicating the presence of other novel *cry* genes.

The *cry* gene content of each isolate had to be determined as one *B. thuringiensis* strain can contain more than one *cry* protein; as such this analysis provided information on the targeted group of insect pests. Carozzi et al. (1991) proposed PCR as an accurate and fast methodology for the identification of novel strains and the prediction of insecticidal activity of new isolates, and they also forecast the possible use of PCR for the discovery of previously unknown *cry* genes. Manuel and Victor (2003) reported that the most common genes found in nature are those with in the *cry1* subfamily, and our observation of the the high share of the *cry1* genes is in agreement with this view. There have also been several reports of a high frequency of certain *cry1* gene combinations (Bravo et al. 1998; Ferrandis et al. 1999; Uribe et al. 2003). A combination of *cry1C* and *cry1D* genes were found in all of the *cry*-positive strains, which conforms with the results of Bravo et al. (1998) and Ferrandis et al. (1999). This *cry1C-1D* linkage may be explained by their common location on the same replicon (Sanchis et al. 1988).

Cry proteins are selectively active against certain insect species. Consequently, strains containing several types of *cry* genes encoding highly active insecticidal crystal proteins might have a wider pest spectrum or increased activity (Seifinejad et al. 2008). Carozzi et al. (1991) determined the biological activity of 28 strains by bioassays and found that the predicted toxicity corresponded with PCR amplification product profiles. Masson et al. (1998) and Ferrandis et al. (1999) found that presence of specific genes is not an accurate indicator of toxicity because the genes could be inactive, under the control of an inefficient promoter or be expressed at a concentration too low to cause toxicity. The bioassay studies of each new isolate on a variety of insect pests are must for its possible use as potential bio-agent.

In conclusion, the results of our investigation indicate that there is considerable phenotypic and genetic diversity among *B. thuringiensis* isolates and that insecticidal activity explains the beneficial microbial resources from root nodules of leguminous crop. *Bacillus thuringiensis* isolates obtained from a number of different legume cultivars may lead to better understanding of their ecological distribution, interaction with other nodule bacteria. Also, these new *B. thuringiensis* isolates would be useful to open new vistas in the area of integrated pest management for sustainable agriculture.

Acknowledgements The facilities provided by the Director, ICAR–Vivekananda Institute of Hill Agriculture, Almora-263601, Uttarakhand, India to carry out this study are gratefully acknowledged.

References

Allwin L, Kennedy JS, Radhakrishnan V (2007) Characterization of different geographical strains of *Bacillus thuringiensis* from Tamil Nadu. Res J Agri Biol Sci 3:362–366

Andrup L, Jensen GB, Wilcks A, Smidt L, Hoflack L, Mahillon J (2003) The patchwork nature of rolling–circle plasmids: comparison of six plasmids from two distinct *Bacillus thuringiensis* serotypes. Plasmid 49:205–232

Aronson AI, Beckman W, Dunn P (1986) *Bacillus thuringiensis* and related insect pathogens. In: Microbiol Rev Ingraham JL (ed) Washington DC, American Society for Microbiology, pp 1–24

Arturo RR, Ibarra JE (2005) Fingerprinting of *Bacillus thuringiensis* type strains and isolates by using *Bacillus cereus* group specific repetitive extragenic palindromic sequence based PCR analysis. Appl Environ Microbiol 71:1346–1355

Arturo RR, Ibarra JE (2008) Plasmid patterns of *Bacillus thuringiensis* type strains. Appl Environ Microbiol 74:125–129

Attathom T, Chongrattanameteekul W, Chanpaisang J, Siriyan R (1995) Morphological diversity and toxicity of delta-endotoxin produced by various strains of *Bacillus thuringiensis*. Bull Entomol Res 85: 167–173

Bai Y, D'Aoust F, Smith DL, Driscoll BT (2002) Isolation of plant-growth-promoting *Bacillus* strains from soybean root nodules. Can J Microbiol 48:230–238

Beatrix T (1997) The difference between conventional *Bacillus thuringiensis* strains and transgenic insect resistant plants: Possible reasons for rapid resistance development and susceptibility of non target organisms. Prepared for the 3rd Meeting of the Open-ended Working Group on Biosafety, Oct 13–17, Montreal, Canada, pp 1–6

Bizzarri MF, Bishop AH (2008) The ecology of *Bacillus thuringiensis* on the Phylloplane: colonization from soil, plasmid transfer, and interaction with larvae of *Pieris brassicae*. Microb Ecol 56:133–139

Bradford M (1976) A rapid and sensitive method for the quantification of micro-gram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–25

Bravo A, Sarabia S, Lopez L, Ontiveros H, Abarca C, Ortiz A, Ortiz M, Lina L, Villalobos FJ, Pena G, Valdez MN, Soberon M, Quintero R (1998) Characterization of *cry* genes in a Mexican *Bacillus thuringiensis* strain collection. Appl Envion Microbiol 64:4965–4972

Burges HD, Hurst JA (1977) Ecology of *Bacillus thuringiensis* in storage moths. J Invertebr Pathol 30:131–139

Burges HD, Jarrett P (1990) Preparation of strains of *Bacillus thuringiensis* having an improved activity against certain lepidopterous pest and novel strain produced thereby. US Patent Number 4: 935353. United States Patent and Trademark Office, Alexandria

Carozzi NB, Kramer VC, Warren GW, Evola S, Koziel MG (1991) Prediction of insecticidal activity of *Bacillus thuringiensis* strains by polymerase chain reaction product profiles. Appl Envion Microbiol 57:3057–3061

Chatterjee SN, Bhattacharya TK, Chandra G (2007) Ecology and diversity of *Bacillus thuringiensis* in soil environment. Afr J Biotechnol 6:1587–1591

Collins CH, Lyne PM (1980) Microbiological methods. Butterworth and Co., London

da Silva RB, Valicente FH (2013) Molecular characterization of *Bacillus thuringiensis* using rep-PCR. SpringerPlus 2:641. doi:10.1186/2193-1801-2-641

Dangar TK, Babu YK, Das J (2010) Population dynamics of soil microbes and diversity of *Bacillus thuringiensis* in agricultural and botanic garden soils of India. Afr J Biotechnol 9:496–501

Dean DH (1984) Biochemical genetics of the bacterial insect-control agent *Bacillus thuringiensis*: basic principles and prospects of genetic engineering. Biotechnol Gen Eng Rev 2:543–525

De Brujin FJ (1992) Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergenic consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium* isolates and other soil bacteria. Appl Environ Microbiol 58:2180–2187

Dulmage HT, Correa JA, Martinez AJ (1970) Co-precipitation with lactose as a means of recovering the spore-crystal complex of *Bacillus thuringiensis*. J Invertebr Pathol 15:15–20

Ferrandis MD, Juarez-Pe'rez VM, Frutos R, Bel Y, Ferre J (1999) Distribution of *cry*I, *cry*II and *cry*V genes within *Bacillus thuringiensis* isolates from Spain. Syst Appl Microbiol 22:179–185

Gonzalez JM, Brown BJ, Carlton BC (1982) Transfer of *Bacillus thuringiensis* plasmid coding for β-endotoxin among strains of *Bacillus thuringiensis* and *Bacillus cereus*. Proc Natl Acad Sci USA 79:6951–6955

Harwood CR, Cutting SM (1990) Molecular biology methods for *Bacillus*. Techniques in microbial genetics, practical manual, vol 56. Department of Entomology, University of Maryland, College Park, pp 1314–1321

Hastowo S, Lay BW, Ohba M (1992) Naturally occurring *Bacillus thuringiensis* in Indonesia. J Appl Bacteriol 73:108–113

Holt JG, Kreig NR, Sneath PHA, Stanley JT, Williams ST (1994) Bergey's manual of determinative bacteriology, 9th edn. The Williams & Wilkins Co, Baltimore

Iriarte J, Porcar M, Lecadet MM, Caballero P (2000) Isolation and characterization of *Bacillus thuringiensis* strains from aquatic environments in Spain. Curr Micobiol 40:402–408

Jaccard P (1908) Nouvelles recherches sur la distribution florale. Bull Soc Vaud Sci Nat 4:223–270

Jarrett P, Stephenson M (1990) Plasmid transfer between strains of *Bacillus thuringiensis* infecting *Galleria mellonella* and *Spodoptera littoalis*. Appl Environ Microbiol 31:346–352

Kan FL, Chen ZY, Wang ET, Tian CF, Sui XH, Chen WX (2007) Characterization of symbiotic and endophytic bacteria isolated from root nodules of herbaceous legumes grown in Qinghai-Tibet Plateau and in other zones of China. Arch Microbiol 188:103–115

Krattiger AF (1997) Insect resistance crops: A case study of *Bacillus thuringiensis* (Bt) and its transfer to developing countries. ISAAA Briefs No 2. ISAAA, Ithaca, p 42

Kronstad JW, Schnepf HF, Whiteley HR (1983) Diversity of locations for *Bacillus thuringiensis* crystal protein genes. J Bacteriol 154:419–428

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Li JH, Wang ET, Chen WF, Chen WX (2008) Genetic diversity and potential for promotion of plant growth detected in nodule endophytic bacteria of soybean grown in Heilongjiang province of China. Soil Biol Biochem 40:238–246

Lupski JR, Weinstock GM (1992) Short interspersed repetitive DNA sequences in prokaryotic genomes. J Bacteriol 174:4525–4529

Manuel P, Victor J-P (2003) PCR-based identification of *Bacillus thuringiensis* pesticidal crystal genes. FEMS Microbiol Rev 26(2003):419–432

Martin BO, Humbert M, Camara E, Guenci J, Walker T, Mitchell P, Andrew M, Prudhomme G, Alloing R, Hukenbeck DA, Morrison GJ, Boulnois JP, Claverys (1992) A highly conserved repeated DNA element located in the chromosome of *Streptococcus pneumoniae*. Nucleic Acids Res 20:3479–3483

Masson L, Erlandson M, Pulstai-Carey M, Brosseau R, Juarez- Perez VM, Frutos R (1998) A holistic approach for determining the

entomopathogenic potencial of *Bacillus thuringiensis* strains. Appl Envion Microbiol 64:4782–4788

Ramalakshmi A, Udayasuriyan V (2010) Diversity of *Bacillus thuringiensis* isolated from Western Ghats of Tamil Nadu State, India. Curr Micriobiol 61:13–18

Rohlf FJ (1998) NTSYSpc numerical taxonomy and multivariate analysis system. Version 2.02i. Exeter Software, Setauket, New York

Sanchis V, Lereclus D, Menou G, Chaufaux J, Lecadet MM (1988) Multiplicity of delta-endotoxin genes with different specificities in *Bacillus thuringiensis aizawai*. Mol Microbiol 2:393–404

Schnepf E, Crickmors N, Rie JV, Lereclus D, Baum J, Feitelson J, Zeigler DR, Dean DH (1998) *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol Mol Biol Rev 62:775–806

Seifinejad A, Jouzani SGR, Hosseinzadeh A, Abdmishani C (2008) Characterization of Lepidoptera-active *cry* and *vip* genes in Iranian *Bacillus thuringiensis* strain collection. Biol Control 44:216–226

Shishir A, Roy A, Islam N, Rahman A, Khan SN, Hoq MM (2014) Abundance and diversity of *Bacillus thuringiensis* in Bangladesh and their cry genes profile. Front Environ Sci 2: 20. doi:10.3389/fenvs.2014.00020

Siliciano SD, Fortein N, Mihoc A, Wisse G, Labelle S, Beaumier D, Ouellette D, Roy R, Whyte LG, Banks MK, Schwab P, Lee K, Greer CW (2001) Selection of specific endophytic bacterial genotypes by plants in response to soil contamination. Appl Envion Microbiol 67:2469–2475

Smith RA, Couche GA (1991) The phylloplane as a source of *Bacillus thuringiensis* variants. Appl Environ Microbiol 57:311–315

Travers RS, Martin AW, Reichelderfer CF (1987) Selective process for efficient isolation of soil *Bacillus* spp. Appl Environ Microbiol 53: 1263–1266

Uribe D, Martinez W, Ceron J (2003) Distribution and diversity of *cry* genes in native strains of *Bacillus thuringiensis* obtained from different ecosystems from Colombia. J Invertebr Pathol 82:119–127

Valicente FH, Lana UGP (2008) Molecular Characterization of the *Bacillus thuringiensis* (Berliner) Strains 344 and 1644, Efficient Against Fall Armyworm Spodoptera frugiperda (J. E. Smith). Revista Brasileira de Milho e Sorgo 7(3):195–209

Versalovic J, Schneider M, De-Bruijn FJ, Lupski JR (1994) Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Methods Mol Cell Biol 5:25–40

Wadman M (1997) Dispute over insect resistance to crops. Nature 388:817

Xue J, Liang GM, Crickmore N, Li HT, He KL, Song FP, Feng X, Huang DF, Zhang J (2008) Cloning and characterization of a novel Cry1A toxin from *Bacillus thuringiensis* with high toxicity to the Asian corn borer and other lepidopteran insect. FEMS Microbiol Lett 280:95–101

Zhang YZ, Chen WF, Li M, Sui XH, Liu HC, Zhang XX, Chen WX (2012) *Bacillus endoradicis* sp. nov., an endophytic bacterium isolated from soybean root. Int J Syst Evol Microbiol 62:359–363