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Characterization of native *Bacillus thuringiensis* strains against *Spodoptera litura* and *Spodoptera exigua*

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ABSTRACT

In the present study 12 native *Bt* strains isolated from insect cadavers were screened for their bioefficacy against neonates of economically important polyphagous pests *Spodoptera litura* and *Spodoptera exigua* by feeding assays at single concentrations of 10 µg/g of diet. Toxicity of *Bt* strains against neonates of *S. litura* and *S. exigua* varies from 39.04% (VKK-EV and VKK-PX2) to 70.97% (VKK-AC1) and 20.0% (VKK-AC1 and VKK-MPS) to 88.00% (VKK-AC2) on 7th day after treatment respectively. The LC_{50} values for potential *Bt* strains against *S. litura* varied from 0.87 µg/gm (VKK-AC1) to 9.36 µg/gm (VKK-AG2) while, against *S. exigua* ranged from 1.00 µg/g (VKK-AC2) to 13.95 µg/g (VKK-SO) of diet. Gene profiling of potential Bt strains revealed the presence of *cry1A*, *cry1D*, *cry1I,* and *cry2* gene. Further studies on characterization of these novel *cry* genes from potential native *Bt* strains will be valuable for management of *Spodoptera* spp.

Keywords: *Bacillus thuringiensis*, Beet armyworm**,** *Cry* gene, Insecticidal activity, Tobacco caterpillar

The tobacco caterpillar *Spodoptera litura* Fabricius is a polyphagous pest known to cause economic damage to more than 150 species of agricultural crops distributed in 44 families worldwide (Kranthi *et al.* 2002). The beet armyworm, *Spodoptera exigua* (Hubner) which was earlier known to infest cotton, jute, tobacco, tomato, cabbage, chilli and alfalfa in India, now became a serious pest of chickpea during the seedling stage (Shanker *et al.* 2014). Till date, control measures for management of this pest mainly focused on spray of insecticides. However, the development of resistance to most insecticides and associated environmental concerns has shifted the focus towards alternative methods of controls which have no negative environmental impacts. Among the various approaches, the major viable alternative is *Bacillus thuringiensis* (*Bt*) which has been successfully used as a bio-insecticide.

Bt is a gram positive, spore-forming, facultative, bacterial pathogen that produces parasporal crystals containing one or more insecticidal crystal (Cry) proteins. Cry proteins are selectively toxic to insects with a great potential to control a number of pests belonging to Lepidoptera, Diptera and Coleoptera and are safe to the environment (Zhong *et al.* 2000). Although *Bt* Cry toxins are effective insecticidal proteins, still a significant number of insects like *Spodoptera* spp are found to be resistant to the commercially available *Bt* toxins (Alotaibi 2013). Globally screening for novel *Bt* strains isolated from various habitats has led to the discovery of strains with toxic activity against a broad range of insect orders (Gao *et al.* 2008, Gorashi *et al.* 2014, Daravath *et al.* 2015, Tripathi *et al.* 2016). Therefore, characterization of native *Bt* strains with novel toxins is of significance for exploring alternatives to the problem of resistance development. The present study was carried out to explore the insecticidal activity of native *Bt* strains isolated from insect cadavers against *S. litura* and *S. exigua* followed by molecular characterization to predict the gene of significance.

MATERIALS AND METHODS

Revival of Bacillus thuringienesis *strains and spore-crystal complex preparation*: Twelve *Bt* strains isolated from insect cadaver and three reference strains, viz. *Bt* subsp. *kurstaki* strains HD1, HD73 and *Bt* subsp. *thuringiensis* HD2 (*Btt*) were retrieved from bacterial stock of Insect Physiology and Molecular Biology Laboratory, Division of Entomology, IARI, New Delhi in 2016. Spore-crystal complex of all the *Bt* strains were prepared as described by Dulmage *et al.* (1970) and was stored in airtight sterile glass vials at 10°C for further use.

Insect rearing and bioassays

Collection and maintenance of test insects: Larvae of *S. litura* and *S. exigua* were collected from cole crop and chickpea field respectively and were reared on chickpea

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based semi-synthetic diet at 27 ±1°C and 60–70% RH. Sex-wise pupae were separated and placed in plastic container bottom lined with blotting paper. The adults that emerged were transferred to mating jars (20 cm height × 15 cm diameter) and offered 10% honey solution throughout their egg-laying period. Paper strips folded in fan like manner were kept inside the mating jar for egg laying. The strips having egg masses were removed every day and kept in separate container. The neonates less than 1 day old were used for bioassays.

Screening bioassays: Twelve *Bt* strains along with three reference *Bt* subsp. *kurstaki* HD-1, HD-73 and *Btt* were evaluated against neonates of both *S. litura* and *S. exigua* for its insecticidal activity. The screening assays were carried out by diet incorporation method at single concentrations of 10 µg/gm of diet on the basis of total protein concentration.10 µl of stock suspension of spore crystal complex (10000 µg/ml) was mixed with 100 µl of double distilled water (DDW) and incorporated in 10 g diet to get a concentration of 10 µg/g. The diet was equally divided and placed in plastic container (2 cm × 5 cm). Each container served as one replicate, with three replications per treatment. Ten neonates were released on the treated diet per replication. Diet in the control was mixed with the same volume of sterilized distilled water. All the assays were conducted under controlled conditions of 27 ± 2°C and 60–70% relative humidity (RH). Mortality data was recorded at an interval of 24 h till 7 days. Per cent mortality was calculated on 4^{th} and 7^{th} day of bioassay.

Virulence bioassays: *Bt* strains which showed >50% mortality in screening assays were used for virulence bioassays. Six concentrations, viz. 0.01, 0.1, 1.0, 5.0, 10 and 25 µg/g were used for virulence bioassay. A minimum of 210 neonates were used for each bioassay and all bioassays were conducted as mentioned above. Mortality data was recorded till 7^{th} day. Mortality data on 7^{th} day was analysed to calculate median lethal concentrations (LC_{50}).

Statistical analysis: The data of mortality on the 4^{th} and 7^{th} day of treatment were corrected with control mortality using Abbott's formula. The mortality data observed on 4^{th} and 7^{th} day of treatment were subjected to analysis of variance (ANOVA) at 5% level of significance using Statistical Analysis System (SAS) version 4.2 (SAS Institute Inc. Cary, USA), to compare the insecticidal activities among different isolates. The significantly different means (<0.05) were separated using tukey's studentized range (HSD) test. The LC_{50} values for virulence bioassays were calculated using maximum likelihood programme (MLP) 3.01 (Ross 2000). The significance of difference between two LC_{50} was determined on the basis of overlap of 95% fiducial limits.

Amplification and characterization of cry genes: Sample of *Bt* strains for PCR was prepared as described by Bravo *et al.* (1998) with few modifications. *Bt* strains were grown for 12 h on Luria agar plates. A loop of cells was transferred to 100 µl of sterile distilled water, and the mixture was frozen for 20 min at -80°C and then transferred to boiling water for 10 min to lyse the cells. The resulting cell lysate was centrifuged (30 s at 10000 rpm) and 10 µl of supernatant was used as a DNA template in the PCR. PCR characterization was performed to identify the toxin-encoding genes using oligonucleotide pairs specific for genes (Table 1) as per Daravath *et al.* (2021)

Table 1 Characteristics of the primer sets used to identify *cry* genes in potential *Bacillus thuringiensis* strains by PCR

cry gene	Primer sequence	AT*	Expected product size (bp)	References
cry1A #	F: 5'-CCGGTGCTGGATTTGTGTTA-3' R: 5'-AATCCCGTATTGTACCAGCG-3'	52	490	Carozzi *et al.* (1991)
cry 1D	F: 5'-TGTAGAAGAGGAAGTCTATCCA-3' R: 5'-TATCGGTTTCTGGGAAGTA-3'	49.4	284	Ceron *et al.* (1995)
cry1I	F: 5'-GCTGTCTACCATGATTCGCTTG -3' R: 5'-CAGTGCAGTAACCTTCTCTTGC-3'	52	1584	Song *et al.* (2003)
cry 2	F: 5'- GTTATTCTTAATGCAGATGAATGGG-3' R: 5'- CGGATAAAATAATCTGGGAAATAGT-3'	47	689-701	Ben-Dov *et al.* (1997)
cry 9	F: 5'- CGGTGTTACTATTAGCGAGGGCGG-3' R: 5'- GTTTGAGCCGCTTCACAGCAATCC-3'	55.5	351-354	
cry 8	F: 5'- ATGAGTCCAAATAATCTAAATG-3' R: 5'- TTTGATTAATGAGTTCTTCCACTCG-3'	48.5	373-376	Bravo *et al.* (1998)
cry 11	F: 5'- TTAGAAGATACGCCAGATCAAGC-3' R: 5'- CATTTGTACTTGAAGTTGTAATCCC-3'	50	305	
cry 20	F: 5'- CAATCCCTGGCTTCACTCGT-3' R: 5'- CCGCGGGCATTAGGATT-3'	49	490	Ejiofar and Johnson (2002)
cry 28	F: 5'- GTATTGGACCGAGGAGATGAAAGT-3' R: 5'- GTACGGCAAAGCGACAGAACA-3'	50	466	

* Annealing temperature; # Presence of *cry1A* in the text means presence of *cry1Aa, cry1Ab* and *cry1Ac.*

RESULTS AND DISCUSSION

Perusal of the mortality data (Fig 1) showed that there was significant variation in the insecticidal activity among the native *Bt* strains. Mortality of *S. litura* neonates varied from 17.24% (VKK-EV) to 44.82 % (VKK-SL1) on 4th day of treatment and 39.04% (VKK-EV and VKK-PX2) to 70.97% (VKK-AC1) on 7th day of treatment. In case of *S. exigua* mortality varied from 13.8% (VKK-AC1) to 65.52% (VKK-AC2) on the 4th day of treatment and 20.0% (VKK-AC1 and VKK-MPS) to 88.00% (VKK-AC2) on 7th day after treatment (Fig 1). All the reference *Bt* strains were found to be less effective against *S. litura* on 4th day as compared to *S. exigua*. However, on 7th day mortality of *S. litura* ranged from 23.33-46.66%, whereas in *S. exigua* it varied from 36.66-43.33% (Fig 1). *Bt* subsp. *kurstak*i strain HD-73 was found to be more effective against *S. litura* and HD-1 against *S. exigua.* However, *Bt* subsp. *thuringiensis* was found to be at par against both insects. Earlier, narrow range of pathogenicity of *Bt* products against species of *Spodoptera*, viz. *S. litura*, *S. frugiperda* and *S. littoralis* was reported (Whitlock *et al.* 1991, Federici 1999). Later on, Prabagaran *et al.* (2002) reported that five *Bt* strains out of 18 had the ability to kill at least 50% second-instar larvae of *S. litura*. Similarly, Manimegalai *et al.* (2005) also reported that *Bt* isolate obtained from cadavers of a silkworm, *Bombyx mori* caused 71.3% mortality of *S. litura.* Hire *et al.* (2009) reported an indigenous Bt strain HD-550 toxic to *S. litura*. However, variability in form of toxins, source of the toxins, and insect stage as well as population, make the results difficult to compare among laboratories. Most of the native strains tested in this study were found to be better than reference *Bt* strains, viz. *Btk* HD1 which is the most widely used strain in commercial formulations to control lepidopteran pests (Fig 1).

The LC_{50} values for potential *Bt* strains against *S. litura* varied from 0.87 µg/gm (VKK-AC1) to 9.36 µg/gm (VKK-AG2) while those against *S. exigua* from 1.00 µg/gm (VKK-AC2) to 13.95 µg/gm (VKK-SO) of diet (Table 2). Out of six common strains (VKK-HA1, VKK-LO, VKK-SO, VKK-AC2, VKK-BB1 and VKK-AG2) two strains VKK-AC2 and VKK-AG2 were found to be more effective against neonates of *S. exigua* than *S. litura* as LC_{50} of VKK-AC2 and VKK-AG2 was found to be 5 and 4.5 fold higher against the neonates of *S. litura* (Table 2 and Fig 2). The variability in susceptibility of these two species of same genus might be due to the variability in gut physiology such as pH, midgut proteases and toxin receptors (de Maggad *et al.* 2003). Molecular characterization based on PCR was performed to identify the toxin-encoding genes using nine oligonucleotide pairs (Table 1). The PCR has been widely used to characterize the Bt strains based on *cry* genes (Bravo *et al.* 1998). Out of 10 Bt strains, *cry1A* gene was amplified in seven strains i.e. VKK-HA1, VKK-BB1, VKK-AC1, VKK-AC2, VKK-LE1, VKK-SO and VKK-EV. In two Bt strain (VKK-HA1, VKK-BB1) *cry1D* is present along with *cry1A* and alone in VKK-AG2. Only VKK-SO amplified *cry1I* along with *cry1A*. Similarly, *cry2* gene was also identified in VKK-AC1 along with *cry1A* and *cry1D*. However, no targeted *cry* gene was detected in VKK-SL1 and VKK-LO. In the present study seven strains (70%) showed the presence of *cry1A* type gene (490 bp) followed by *cry1D* type genes (284 bp) in 30% strains. Similarly, many previous studies also reported that *cry1* type genes were most abundant in

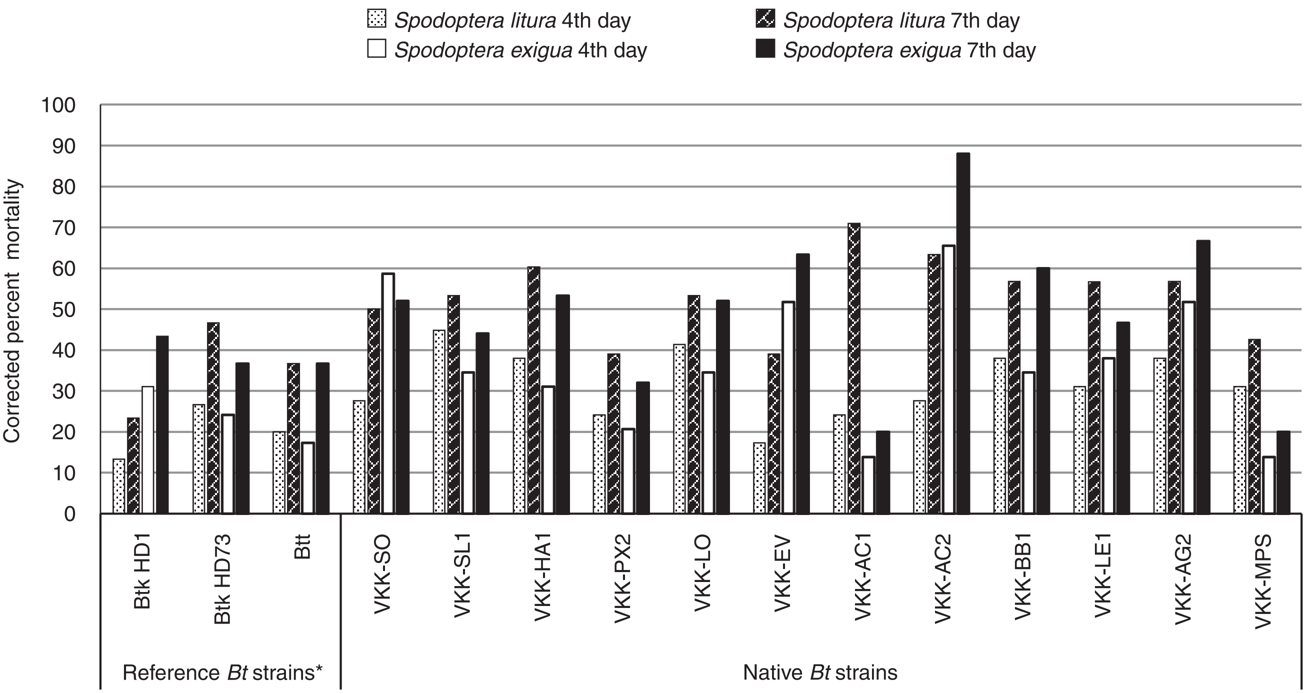

Fig 1 Efficacy of native *Bt* strains along with three reference strains against neonates of *Spodoptera litura* and *Spodoptera exigua* on 4th and 7th day after treatment in terms of percent mortality.

Table 2 Comparative toxicity of potential native *Bacillus thuringiensis* strains against neonates of *Spodoptera litura* and *Spodoptera exigua*

Bacterial strain ID	LC_{50} (µg/g of diet on 7th day)	95 % Fiducial limit		Slope ± Standard error	Chi square	Degrees of freedom
		Lower	Upper			
Spodoptera litura						
VKK-HA1	4.27	1.23	26.85	0.40±0.11	1.81	4
VKK-LO	8.54	3.84	28.22	0.69±0.18	2.45	4
VKK-SO	6.99	2.81	26.87	0.59±0.15	9.15	4
VKK-AC2	5.66	2.64	13.94	0.73±0.16	3.90	4
VKK-BB1	4.11	1.75	10.59	0.66±0.15	8.11	4
VKK-AG2	9.36	2.64	13.94	0.39±0.16	3.90	4
VKK-SL1	5.44	2.08	20.42	0.53±0.12	5.03	4
VKK-LE1	5.18	2.19	11.32	0.84±0.24	10.68	4
VKK-AC1	0.87	0.27	2.39	0.45±0.10	4.57	4
Spodoptera exigua						
VKK-HA1	9.48	3.24	65.35	0.48±0.12	3.07	4
VKK -LO	13.68	5.93	80.92	0.67±0.20	4.60	4
VKK -SO	13.95	4.37	118.09	0.41±0.09	2.36	4
VKK-AC2	1.00	0.37	2.32	0.60±0.12	11.76	4
VKK-BB1	3.16	1.16	10.23	0.71±0.11	3.47	4
VKK-AG2	2.06	0.45	12.65	0.34±0.09	3.01	4
VKK-EV	2.06	0.84	4.68	0.66±0.13	8.84	4

native *Bt* strains isolated from different habitats (Salama *et al.* 2015, Jain *et al.* 2017). *cry1I* and *cry2* genes were present in only one strain each and that too in combination with *cry1A* (VKK-SO) and *cry1A+cry1D (*VKK-AC1) respectively. VKK-AC2 having *cry1A* alone found to be most effective *S. exigua* with lowest LC_{50} (1.00 µg/g of diet) followed by VKK-AG2 (LC_{50}= 2.06 µg/g of diet) having *cry1D* alone. Moreover, VKK-AC1 contained three genes, i.e. *cry1A, cry1D* and *cry2* and found to be most effective against neonates of *S. litura* with lowest LC_{50} (0.87 µg/g of diet) but not against *S. exigua*. Similarly, Prabagaran *et al.* (2002) reported the presence of *cry1* genes in different indigenous *Bt* strains potentially active against *S. litura*. Reddy *et al.* (2013) have reported a novel

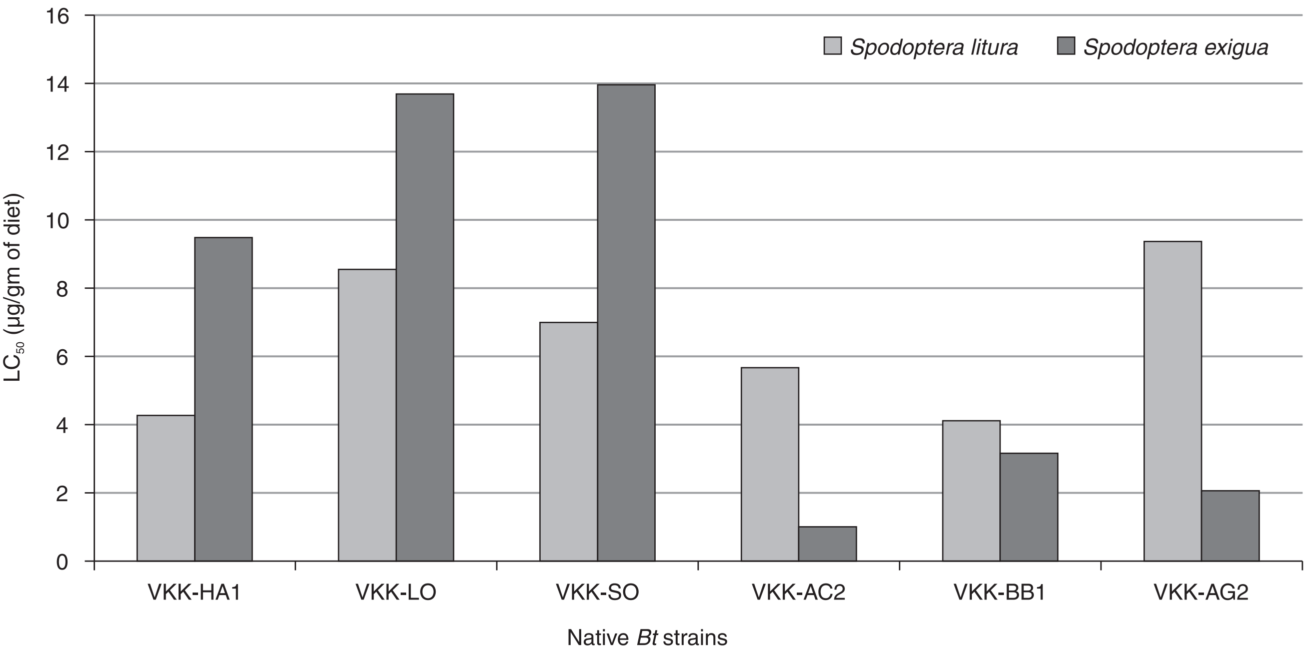

Fig 2 Comparative toxicity (LC_{50}) of six common potential native *Bacillus thuringiensis* strains against neonates of *Spodoptera litura* and *Spodoptera exigua.*

cry1 gene from *Bt*-1 DOR isolate effective against *S. litura* and other lepidopteran pests.

Overall activity spectrum of *Bt* strain is a function of additive and/or synergistic interactions of individual Cry proteins present in their proportional amounts. Most of *Bt* strains have the same basic toxin structure, but differ in insect host range, perhaps because of different degree of binding affinity to the toxin receptors in the insect gut. Ever since the cloning of first *cry* gene (*cry1Aa*) from *Btk* HD-1, these insecticidal crystal protein genes are the major source for the development of insect-resistant transgenic plants (Romeis *et al.* 2006). In 2018, global area of biotech crops was 191.7 million ha of which 23.7 mh produce insecticidal proteins of *Bt* for the control of pests (ISAAA 2018). But still in spite of the variability of Cry proteins and the range of susceptible organisms, a significant number of insects like *Spodoptera* spp that cause great losses on crop production are not susceptible to the commercially available *Bt* toxins.

Thus, in the present study, we characterize potential native *Bt* strains isolated from insect cadavers in order to find novel strains toxic against *S. litura* and *S. exigua,* which are known to be tolerant to most of the known Cry toxins. Further studies on characterization of these novel *cry* genes from potential native *Bt* strains will be valuable for management of *Spodoptera* spp. Recently, Huang *et al.* (2020) used CRISPR-mediated knockouts to evaluate the role of five genes encoding candidate *Bt* toxin receptors against three *Bt* cry toxins (Cry1Ac, Cry1Fa, and Cry1Ca) in *S. exigua.* Present study found that six native *Bt* strains found to be effective against neonates of both *S. litura* as well as *S. exigua*. Thus, reveals the usefulness of screening studies of novel *Bt* strains. These strains can be further utilized for studying insecticidal activity against other important insect pests as well as for advance gene profiling for developing insect resistant plants or developing formulation by optimizing the production conditions and applied for the management of these pests.

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