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RESEARCH ARTICLE

Prospecting in Western Ghats of Karnataka for indigenous *Bacillus thuringiensis* isolates harbouring novel crystal toxin genes for sugarcane pest management

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Abstract
Prospecting for potential novel *Bacillus thuringiensis* with new holotype crystal toxins was carried out in the Western ghats hill range of Karnataka state, India. From the soil samples collected three *Bt* isolates SBIKWG 12, SBIKWG 24 and SBIKWG 70 were isolated. Of these while the two isolates, namely SBIKWG 12 and SBIKWG 24 produced bipyramidal crystal toxins, the third isolate produced spherical crystal. PCR screening of the isolates revealed the presence of lepidopteran and coleopteran active *cry* genes. Partial sequences obtained from these isolates revealed the presence of multiple crystal toxin genes. BlastX analysis of the partial gene sequences indicated the potential for the occurrence of new holotype crystal toxin genes in SBIKWG 24 and SBIKWG 70.

Keywords: *Bacillus thuringiensis;* Borers; Coleoptera; *cry1* gene; *cry8* gene; Holotypes; Lepidoptera; Sugarcane; India

Introduction

Lepidopteran stem borers are among the major insect pests of sugarcane all over the world. Several species of moth borers belonging to different genera attack sugarcane worldwide (Long and Hensley 1972; Goebel and Sallam 2011; Srikanth et al. 2016) causing significant yield losses of nearly 25-30 percent (Kalunke et al. 2009). Moth borers are difficult to control with insecticides due to their inaccessibility and hidden nature of larval feeding inside the cane. Biological control has been the main component of their management in countries like India (Srikanth et al. 2016) despite limitations in providing efficient control of borers (Mukunthan et al. 2003). Among the coleopteran pests white grub *Holotrichia serrata* F. is a serious in sugarcane causing up to 80-100 per cent damage (David and Ananthanarayana 1986). The subterranean habit makes this pest intractable for control and the available biological agents such as the entomopathogenic fungus *Beauveria brongniartii* (Sacc.) Petch (Srikanth et al. 2010) and entomopathogenic nematode *Heterorhabditis indicus* (Sankaranarayanan et al. 2006) have limited efficacy for various reasons. Insect resistant sources are not available for *H.serrata* F. in sugarcane germplasm collections of the world which limits the pursuit of insect resistance breeding program.

In the above scenario, the advancements made in the biotechnology of the bacterium *Bacillus thuringiensis* (*Bt*) offer to develop insect resistant crop plants, including sugarcane through genetic engineering. Thousands of *Bt* strains been isolated across the world to a wide range of insect orders, nematodes, mites and protozoans. The International Committee on *Bt* Toxin Nomenclature has recently revised the nomenclature system of this toxin proteins which was earlier classified 78 (Cry1 - Cry78*)* different types of Cry proteins (Crickmore

et al. 2020). *Cry1, Cry2* and *Cry9* groups exhibit strongest activity against lepidopteran insects; *Cry3, Cry7* and *Cry8* groups are most toxic to coleopteran insects whereas *Cry4* and *Cry11* are most toxic to dipteran insects; the host spectrum of other Cry toxins has also been reviewed well (Van Frankenhuyzen 2013; Palma et al. 2014; Crickmore et al. 2020). Cry1 toxins are the largest and best known family used against lepidopteran pests worldwide either as biological insecticides or for developing insect resistant transgenic crops (Sanchis 2011). ICAR-Sugarcane Breeding Institute (ICAR-SBI), Coimbatore, India, carried out pioneering work on the development and evaluation of insect resistant transgenic sugarcane varieties with *cry1A* genes against borers in India (Christy et al. 2009; Arvinth et al. 2010). Despite the availability of several approaches in transgenic research (Srikanth et al. 2011), the fundamental requirement is the identification and use of novel genes which are free from any intellectual property issues.

Many researchers have prospected for novel *Bt* and crystal toxin genes from biodiversity rich hot spots like Nilgiri Bioshpere of Western ghats (hill range) in Tamil Nadu (Ramalakshmi and Udayasuriyan 2010), Western ghats (Mahadeva Swamy et al. 2013), and other parts of Karnataka state (Pooja et al. 2013), Andaman and Greater Nicobar island (Mahadeva Swamy et al. 2011; Asokan et al. 2013), and sugarcane ecosystem in Tamil Nadu (Singaravelu et al. 2013a, 2013b). Only three primary (*cry8Sa1*, *cry32Aa1* and *cry52Ca1*) and two secondary (*cry2Ai1* and *cry2Al1*) holotype *Bt* crystal toxin genes from India are recognized by the International Committee on *Bt* Toxin Nomenclature. Of these the sequence of the holotype *cry8Sa1*,(NCBI Accession No.JQ740599) and the genome sequence of isolate (*Bt 62*) carrying the gene were reported by us in recent studies (NCBI genome Accession. No. SRP1275532 for chromosome and SRP 129858 for plasmid). Since analysis of the partial sequences or *cry* genes obtained from *Bt* collected in Western ghats of Karnataka and deposited in NCBI by Mahadeva Swamy et al. (2013) revealed the presence of novel crystal toxin genes in the western ghats of Karnataka, we undertook a survey in the region to isolate novel indigenous *Bt* isolates harbouring holotype *cry* genes for use against sugarcane pests.

Materials and Methods

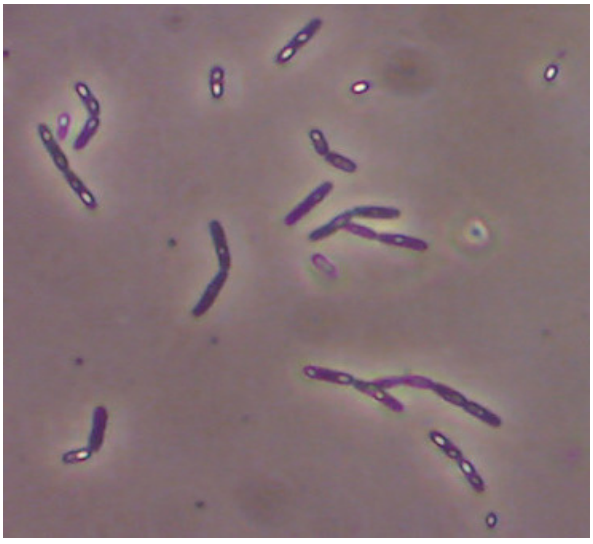
Soil samples collection

For isolation of *Bt*, soil samples were collectedfrom of Western ghats in Udupi, Dakshina Kannada,Chikamaglur and Shivamoga districts of Karnataka. Soils samples from diverse crop systems, viz. arecanut, coffee, rubber plantations, paddy fields, forest areas in Kundadri hills, Agumbe and Charmady ghat were collected by scraping off surface soil up to a depth of 5 -10 cm with a spatula. Samples were collected in plastic bags, labelled in the field, transferred to the lab and stored at room temperature.

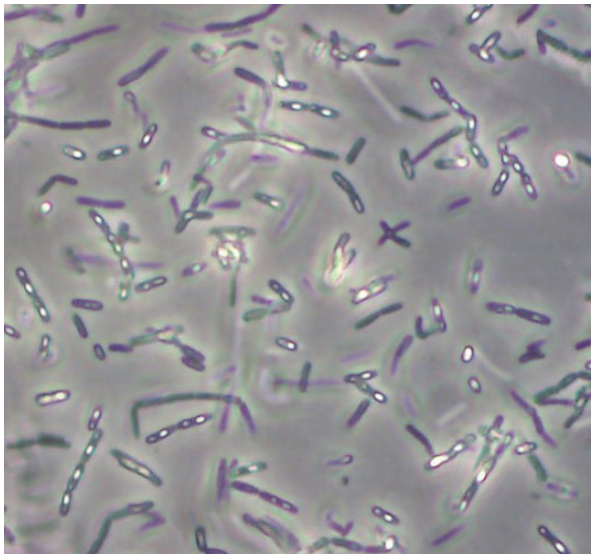
Bt isolation

Isolation of *Bt* from soils was carried out with a slight modification to the method followed by Travers et al. (1987). Ten grams of soil sample was suspended in 100 ml of 0.85% NaCl solution and heated at 80°C for 15 min in a water bath. One ml of the heat treated sample was inoculated in 50 ml of Luria Bertani (LB) broth and incubated overnight at 30°C with an orbital shaking of 250 rpm. Serial dilution (10^{-1} to $10^{-6)}$ of the broth was performed and each dilution was plated in Travers (T3) medium. The plates were then incubated at 30°C for 48 h. After incubation, *Bt* like colonies were selected and streaked on T3, and incubated at 30°C for 72 h. Single colonies were observed under phase contrast microscope for identification

(a)



(b)



(c)

Figure 1. Phase contrast microscope images of Bt isolates from western ghats (a) SBIKWG 12 (100x magnification) ; (b) SBIKWG 24 (40x magnification) ; (c) SBIKWG 70 (40x magnification)

of *Bt*. Isolates showing the presence of crystalline inclusions were selected as *Bt* and streaked on T3 agar medium for single colony purification. Broth culture (pH adjusted to 6.9) was obtained from the isolated single colonies of crystal positive *Bt* isolates. Glycerol stocks of *Bt* isolates were prepared by using equal amounts of 30% glycerol and 72 h old T3 broth culture and stored at -20°C for further studies.

PCR screening for cry genes

Polymerase chain reaction (PCR) was used to identify *cry* gene types of *Bt* isolates. *cry1,cry8* and *cry9* gene positive reference *Bt* strain (HD1), Bt 62, 4AT1 and indigenous *Bt* isolates from soils of western ghats were streaked on Luria Agar (LA) plate and grown overnight at 30°C. Reference strains of *B. thuringiensis* for *cry1* and *cry9* genes used in this study was obtained from Bacillus Genetic Stock Centre (BGSC), Ohio, USA (Table 1). For screening of cry8 genes Bt 62 isolated by ICAR-Sugarcane Breeding Institute (ICAR-SBI), Coimbatore, India was used. Two swipes of 12 h old cultures were suspended in 200 µl of sterile distilled water and the isolates were heated by placing them in boiling water for 10 min. The lysed cells were allowed to settle for 8-10 min at room temperature and the supernatant was taken as DNA template for PCR reaction. Universal primers (forward and reverse) of *cry1, cry8 and cry9* gene were used to amplify a specific fragment and the amplicon size produced by universal primer for each of the *cry* gene is presented in Table 2.

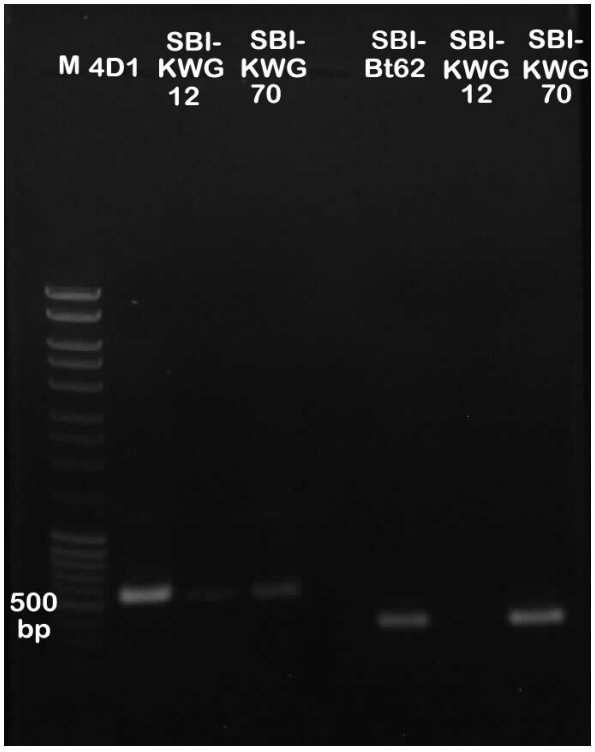


Figure 2. PCR amplification of *cry1* and *cry8* gene of indigenous Bt isolates SBIKWG 12 and SBIKWG 70 from western ghats of Karnataka. M; DNA marker; 4D1 *cry1* gene positive reference Bt isolate; SBI-Bt 62 *cry8* gene positive reference *Bt* isolate

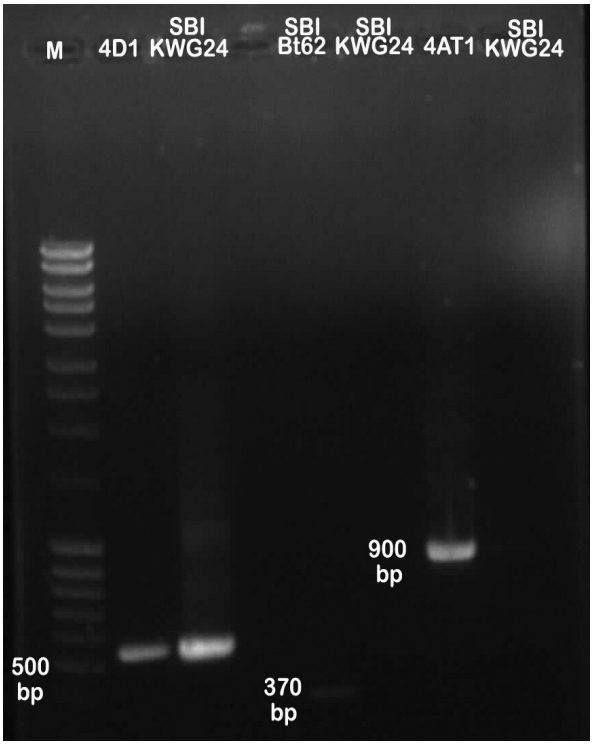


Figure 3. PCR amplification of *cry1,cry8* and *cry9* gene of indigenous Bt isolate SBIKWG 24 from western ghats of Karnataka. M; DNA marker; 4D1 *cry1* gene positive reference Bt isolate; SBI-Bt 62 *cry8* gene positive reference Bt isolate ; 4AT1 *cry9* gene positive reference *Bt* isolate

All PCR reactions were carried out in 50 µl reaction volumes.Twenty µl of template DNA was mixed with reaction buffer containing 1.25 µl of 2.5 mM deoxynucleotide triphosphate mix, 0.5 µl of 10 µM (direct and reverse) primers, 5 µl Taq buffer (10 X) and 1 U of Taq DNA polymerase. Amplifications were carried out in a DNA thermal cycler (Biorad S1000). The PCR conditions for screening the isolates were as follows: single denaturation step at 94°C for 5 min, a step cycle program set for 30 cycles (with a cycle consisting of denaturation at 94°C for 1 min, annealing at 50°C for 30 sec and extension at 72°C for 45 sec), and an extra step of extension at 72°C for 7 min after completion of all the cycles. All PCR reactions were performed with the *cry1* gene positive reference strain *B. thuringiensis* serovar *kurstaki* (HD1), *cry8* gene reference Bt 62 and

Table 1. List of reference *Bt* strains used in this study for PCR screening of respective crystal toxin gene

S.No	Strains	BGCS Code	Genes
1	*B. thuringiensis* subsp *kurstaki* HD1	4D1	*cry1*
2	*B.thuringiensis Bt62*	Isolated from ICAR-SBI,Coimbatore	*cry8*
3	*B. thuringiensis* subsp *japonensis* T23 001	4AT1	*cry9*

Table 2. Universal primers used for screening various crystal toxin genes

S. No.	Sequence	Size (bp)	Targeted gene	NCBI Accession	Reference
1.	5'-CTGGATTTACAGGTGGGGATAT-3'(F) 5'TGAGTCGCTTCGCATATTTGACT-3'(R)	558	*cry1*	M11250 M73250	Bravo et al. (1998)
2.	5'ATGAGTCCAAATAATCTAAATG-3'(F) 5'TTTGATTAATGAGTTCTTCCACTCG(R)	373	*cry8*	U04364 U04365	Bravo et al. (1998)
3	5'-ATGAATCGAAATCATCAAAAT-3'(F) 5'-GTCCCATCTGGATATTGTCG-3'(R)	902	*cry9*	AY971349 GQ249297	Present study

cry9 reference *Bt* strain AT1. Following the amplification, electrophoresis of each PCR sample was done on 1 % agarose-ethidium bromide gel.

Results and Discussion

Bt isolates were identified through phase contrast microscopy from the soil samples collected in sugarcane ecosystem. *Bt* isolates are generally identified by their crystal toxins which are easily visible under phase contrast microscope. In our study, the three *Bt* isolates viz., KWGSBI 12 (Fig.1a), KWGSBI 24(Fig.1b) and KWGSBI 70(Fig.1c) were isolated from 100 soil samples collected from the Western ghats of Karnataka resulting in an isolation percent of 3%. The details of the name of isolate, place of collection, GPS coordinates and crystal toxin shapes are given in Table 3.

The amplicon size produced by universal primer for each of the *cry* gene is given in Table 2. The results of the PCR screening of SBIKWG-Bt12 and SBIKWG-Bt70 *Bt* isolates against *cry1* and *cry8* reference gene is presented in Fig. 2. While isolate KWGSBI-Bt12 was found positive for *cry1* gene and negative for *cry8,* isolate KWGSBI-*Bt70* was positive for both *cry1* and *cry8* genes. PCR screening SBIKWG-Bt24 isolates against *cry1, cr*y8 and *cry9* reference gene is presented in Fig 3. Isolate KWG24 was found positive for *cry1* gene and it was negative for *cry8* and *cry9* gene

Screening of the *Bt* isolates identified in this study with *cry1* gene universal primer revealed the presence of *cry1* gene in *Bt* isolates SBIKWG-Bt12 and SBIKWG-Bt24. Similarly screening with universal cry8 primer revealed the presence of *cry8* gene in SBIKWG-Bt70 showed an amplicon of approximately 370 bp (Fig. 2). When the PCR amplicons of the *cry1* gene positive isolates SBIKWG-Bt24 and cry8 positive isolate SBIKWG-Bt70 were sequenced, we found that the sequencing chromatogram yielded overlapping peaks for both these isolates. Since the conserved sequences of *cry1* and *cry8* genes were used as primers for the screening of the *Bt* isolates, the overlapping peaks observed in the sequencing chromatogram were due to the presence of multiple *cry1* and *cry8* subfamily genes in SBIKWG-Bt24 and SBIKWG-Bt70 respectively. Blastx results of the partial sequence of the cry1 genes of SBIKWG-Bt24 showed a similarity of 87.67%, (NCBI accession ARV85538) 73.61% (NCBI accession AIW52616) and 67.12% (NCBI accession WP087976784) for the top three hits. The top three hits of Blastx results for the *cry8* partial sequence of SBIKWG-Bt70 showed similarity of 54.29 % (NCBI accession OTX96001, WP_076775865, AEZ02302 and ADQ73629). As per the *Bacillus thuringiensis* Toxin Nomenclature Committee

Table 3. Details of *Bacillus thuringiensis* isolates identified in this study

S.No	Name of *Bt* isolate	Place of collection	GPS coordinates		Crystal shape
			Latitude	Longitude	
1.	SBIKWG-Bt12	Bajagoli Karkala Taluk Udupi District, Karnataka	N 13^0 12' 18.55"	E 075^0 04' 29.47"	Bipyramidal
2.	SBIKWG-Bt24	Kabinabaagil Belthangadi Taluk Dakshina Kannada District, Karnataka	N 13^0 01' 54.86"	E 075^0 23' 04.62"	Bipyramidal
3.	SBIKWG-Bt70	Anandur Thirthahalli Taluk Shimoga District Karnataka	N 13^0 31' 30.62"	E 075^0 09' 47.95"	Spherical

classification, a novel toxin is given a four-rank name depending on its degree of pairwise amino acid identity to previously named toxins. Arabic numbers are used for the primary and quaternary ranks, and uppercase and lowercase letters are assigned for the secondary and tertiary ranks, respectively. Genes encoding crystal toxins that share less than 45% pairwise identity are assigned a different first rank (an Arabic number, e.g., *cry1* and *cry2*); two *Cry* proteins sharing less than 78% pairwise identity are assigned a different secondary rank (a capital letter, e.g., *cry1A* and *cry1B*); Cry proteins sharing less than 95% pairwise identity are assigned a different tertiary rank (a lowercase letter, e.g., *cry1Aa* and *cry1Ab*); and, finally, to differentiate between proteins sharing more than 95% pairwise identity, a quaternary rank is assigned (an Arabic number, e.g., *cry1Aa1* and *cry1Aa2*). According to the system established by the committee the pairwise amino acid identity of the isolates SBIKWG-Bt24 and SBIKWG-Bt70 qualify these isolates to be carrying holotype crystal toxin genes. However pair wise amino acid identity data of the full coding sequence of the *cry1* and *cry8* genes needs to be deduced by performing a whole genome sequence of these isolates. Since both the isolates produce overlapping peaks in the sequencing chromatogram, the whole genome sequence will be a rapid way to elucidate the multiple crystal toxin gene composition of the isolates. Once the full coding sequences are deduced and functional validation of these crystal toxin genes against lepidopteran and coleopteran pests of sugarcane is established, development of insect resistant sugarcane transgenics can be made feasible.

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