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Molecular Characterization and Genetic Diversity of Insecticidal Crystal Protein Genes in Native *Bacillus thuringiensis* Isolates

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Abstract The Western Ghats of Karnataka natural ecosystem are among the most diverse and is one of the eight hottest hotspots of biological diversity in the world, that runs along the western part of India through four states including Karnataka. *Bacillus thuringiensis* (*Bt*) strains were isolated from soils of Western Ghats of Karnataka and characterized by molecular and analytical methods as a result of which 28 new *Bt*-like isolates were identified. *Bt* strains were isolated from soil samples using sodium acetate selection method. The morphology of crystals was studied using light and phase contrast microscopy. Isolates were further characterized for insecticidal *cry* gene by PCR, composition of toxins in bacterial crystals by SDS-PAGE cloning, sequencing and evaluation of toxicity was done. As a result 28 new *Bt*-like isolates were identified. Majority of the isolates showed the presence of a 55 kDa protein bands on SDS-PAGE while the rest showed 130, 73, 34, and 25 kDa bands. PCR analysis revealed predominance of Coleopteran-active *cry* genes in these isolates. The variations in the nucleotide sequences, crystal morphology, and mass of crystal protein(s) purified from the *Bt* isolates revealed genetic and molecular diversity. Three strains containing Coleopteran-active *cry* genes showed higher activity against larvae *Myllocerus undecimpustulatus undatus* Marshall (Coleoptera: Curculionidae) than *B. thuringiensis* subsp. *Morrisoni*. Results indicated that *Bt* isolates could be utilized for bioinsecticide production, aiming to reduce the use of chemical insecticide which could be useful to use in integrated pest management to control agriculturally important pests for sustainable crop production.

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Introduction

Despite the isolation and characterization of a relatively large number of different insecticidal proteins to date, there is a need for identification, isolation, and characterization of new insecticidal proteins. The reasons for this are manifold. First, due to the specificity of insecticidal proteins toward particular groups of target pests (host insect spectra), there is a need to clone genes encoding proteins with different spectra of activity, so that for different crops and different geographic regions suitable proteins for combating insect pests are available. The spectra of activity of *Bt* Cry proteins, for example, is mostly limited. Identification of toxins with specificity toward different target insects remains desirable. Second, after prolonged use in one geographic region, insects are known to have the capacity to develop resistance toward chemical insecticides and microbial sprays (for example based on *Bt* spore–crystal mixtures), and are believed to have the capacity to develop resistance toward plants expressing insecticidal proteins. The development of resistance within insect

populations could render existing insecticidal proteins ineffective, creating a need for novel genes and proteins. Third, for health and environmental reasons, it is desirable to identify proteins with high, specific insecticidal potency, and acute bioactivity toward target insect species [1].

Toxins of Cry1, Cry3, Cry7, Cry8, Cry9, Cry14, Cry18, Cry22, Cry23, Cry34, Cry35, Cry36, Cry37, Cry43, Cry55, Cyt1, and Cyt2 groups designate crystal activity against some pests of the order Coleoptera. Crystalline proteins belonging to Cry 1, 2, 4, 10, 11, 16, 19, 20, 24, 27, 32, 39, 44, 47, 48, and 49 may be toxic for dipterans insects. Cry23 and Cry11 are active against hemipteran insects. Pests of Lepidoptera may be susceptible to protein Cry1, Cry8, Cry9, Cry15, Cry22, Cry32, and Cry51. Proteins Cry2, Cry3, and Cry22 are toxic to insects of the order Hymenoptera [2]. *Bt* crystals activity can also be predicted by characterization of *cry* gene profile [3, 4]. Analysis of *cry* gene and crystalline toxins patterns could lead to selection of effective strains that may be employed in production of a new insecticide and be a source of novel genes for bioengineered crop protection [5]. Therefore, the search for and characterization of novel *Bt* strains and genes with varied/novel insecticidal activity is important. The search for strains of *Bt* with novel insecticidal activity has a much higher success rate compared to screening chemical compounds with similar properties. Keeping this information in view, an attempt has been made to isolate *Bt* from regions of Western Ghats and to identify their insecticidal genes with the objectives (i) To isolate *Bt* from Western Ghats region of Karnataka, India. (ii) To identify the spectrum of insecticidal genes of the native isolates. The characterization methods included the determination of crystal protein composition by microscopy, insecticidal *cry* gene determination by PCR and SDS-PAGE method to determine the composition of toxins in bacterial crystals, cloning and sequencing to determine diversity and finally toxicity analysis.

Materials and Methods

Collection of Samples and Isolation of *B. thuringiensis* Strains

Forest areas unaffected by anthropogenic activities were selected at sites situated along Western Ghats of Karnataka, India. Sites were at 30–40 km of distance from each other. 100 g of soil sample was taken from 5 cm depth, after gently removing the top debris in the top soil using a sterile spatula and placed immediately inside the sterile polythene covers (10 × 5 cm). Labels containing the details on date of collection, place of collection, collector's name and description of the place of collection in which the sampling was carried out, were written using a permanent marker and placed inside the polythene cover and secured properly (Supplementary data 1). The soil samples were stored at 4 °C until further use. There was no previous history of use of *Bt* in the soil sampling area. The 1 g of soil sample was suspended in 10 ml of sterile distilled water (10^{-1}) in a boiling tube. The boiling tube was subjected for heat treatment at 650 °C for 30 min and allowed to settle. The 1 ml of heat treated suspension was taken and added to 4 ml of saline (0.85 % NaCl), which gives 5^{-1} dilution. Similarly, dilutions were prepared up to 5^{-5}. The 1 ml aliquots of dilutions 10^{-1}, 5^{-1}–5^{-5} were taken in six different petri plates over which melted T3 agar medium [6] was poured and mixed clockwise and anticlockwise directions. The plates were incubated at 300 °C for 2–3 days. From each soil samples, around 12 colonies resembling *Bt* were selected and subcultured as ribbon streak (four colonies per plate) on T3 agar medium. After 48 h of incubation, smear is prepared from ribbon streak cultures on glass slide and heat fixed. After heat fixing, drops of the Coomassie Brilliant Blue stain (0.133 % Coomassie Brilliant Blue G-250 in 50 % acetic acid) were added and kept as such for 1 min. Then, the smear is washed gently in running tap water. After blot drying with blotting paper, the stained cultures were observed through bright field microscopy for the presence of crystalline inclusions. The isolates showing the presence of crystalline inclusions were selected as *Bt* and streaked on T3 agar medium for single colony purification. Broth culture was made from the isolated single colonies of crystal positive *Bt* isolates. Glycerol stocks were prepared using 24 h old broth culture and stored at −800 °C for further studies. *Bt* isolation index was worked out by dividing the population of crystalliferous *Bt* isolates by the total population of *Bacillus* for each sample collected. *B. thuringiensis* isolates were cultured in glucose yeast salt (GYS) medium (0.08 g/l $CaCl_2$ 2 g/l yeast extract, 0.5 g/l K_2HPO_4, 2 g/l $(NH_4)_2SO_4$, and 1 g/l glucose) until they completed sporulation and autolysis [7]. The spore–parasporal crystal mixtures were thoroughly washed with washing buffer (1 M NaCl containing 0.01 % Triton X-100) and sonicated to separate the spore and parasporal crystals. Samples were centrifuged at 12,000×*g* for 10 min. The pellet was resuspended in sample buffer and boiled for 10 min. SDS-PAGE was performed using 10 % running and 4 % stacking gels [8]. Following electrophoresis, the gels were stained in 0.1 % Coomassie Brilliant Blue G-250. The molecular masses of the parasporal body proteins were estimated by comparison with standard protein molecular weight marker.

Determination of *cry* Genes by PCR

The presence of *cry* genes in native *Bt* strains was characterized with general and gene-specific primers through PCR. Primers were designed according to *cry*-type gene sequences from NCBI GenBank (http://www.ncbi.

nlm.nih.gov/nuccore) using Fast PCR, IDT DNA oligoanalyzer, primer 6 and Blast software (Supplementary data 2). The Oligonucleotides were synthesized at Sigma Aldrich Pvt. Ltd., USA as specified by manufacturer guidelines. PCR was conducted using 250 ng of total *B. thuringiensis* DNA with 2.5 U of Taq DNA polymerase, 200 nM each dNTP, 1 mM each primer, 3 mM $MgCl_2$ in a final volume of 50 µl. Amplification was done in a Applied Biosystems thermal cycler under the following conditions: 5 min of denaturation at 94 °C followed by 25 cycles of amplification with a 1 min denaturation at 94 °C, 45 s of annealing at 45 °C, and 2 min of extension at 72 °C. An extra extension step of 10 min at 72 °C was added after completion of the 25 cycles [9]. PCR products were analyzed on 1 % agarose gel electrophoresis with ethidium bromide (0.5 µg/ml) in TAE buffer (45:1) at 6 V/cm. Nucleotide and deduced amino acid sequences were analyzed with the Blast tools (www.ncbi.nlm.nih.gov/BLAST). BioEdit (version 7.0.4.1) was used for sequence editing and analysis. PCR amplified products were ligated to the TA cloning vector pTZ57R/T, which was used for transforming Escherichia coli DH5α by standard protocols [10] using Fermentas DNA ligation kit. The transformed cells (60 µl) were spread on LB agar plates containing X-gal (270 lg/ml), IPTG (120 µg/ml) and ampicillin (100 µg/ml). The plates were then incubated overnight at 37 °C to screen blue and white colonies. Cloning was confirmed by colony PCR and recombinant plasmid comparison with colony without insert. Finally, cloned fragments were sequenced. BLAST X (version 2.2.6) was used for DNA sequence analysis. Each gene was sequenced at least three times, and a consensus sequence was obtained. Known Cry sequences were obtained from the non-redundant protein database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The sequences of more than 50 Cry proteins were used. Sequence alignments were generated with the CLUSTAL X software program (version 1.8).

Nucleotide Sequence Accession Numbers

The partial nucleotide sequences of the Coleopteran, Hemipteran, and Nematode active *cry* gene obtained from soils of Western Ghats of Karnataka, India were submitted to NCBI GenBank database (http://www.ncbi.nlm.nih.gov/genbank/submit.html).

Bioassays

Preliminary bioassay using highly concentrated spore–crystal suspensions of strains containing coleopteran-active *cry* genes, were performed with the *Myllocerus undecimpustulatus undatus* Marshall (Coleoptera: Curculionidae) using first instar larvae. For each treatment, 10 first instar larvae were used (three replicates per treatment). *B.thuringiensis* subsp. *Morrisoni* was used as positive control. Negative controls for all the insects tested were included using same conditions but without any toxin. Bioassays were conducted at 25 °C in 60–70 % relative humidity with a 16:8 light/dark cycle. The percentage of mortality was scored after 5 days in comparison with parallel control. The isolates with high toxicity were selected for more precise bioassays. The solubilization buffer and water were used as negative controls. The 50 % lethal concentrations and confidence limits were obtained by probit analysis.

Results

The *Bt* strain collection assembled in this study came from soils, collected from undisturbed ecosystems of Western Ghats of Karnataka, India. In total, 28 crystal-forming *Bt* strains were identified. Crystal-forming isolates were selected for detection of different Coleopteran-, Hemipteran-, and Nematode-specific *cry* genes. The strains were characterized by different methods: (I) Microscopic observation of crystals (Supplementary data), (II) PCR to identify Coleopteran-, Hemipteran-, and Nematode-specific active *cry* genes, (III) SDS–PAGE of spore–crystal suspensions to determine the protein profiles of the isolates, (IV) Cloning and sequencing, (V) toxicity test.

Isolation of *Bt* Strains from Soil Samples of the Western Ghats and Morphology of Crystalline Inclusions

Based on the capacity of producing parasporal crystal, detected the presence of *Bt* in 56.0 % of the samples. Isolation index 0.25 was obtained (Supplementary data 3). A total of 391 *Bacillus*-like colonies were subcultured and subjected to microscopic observations for the presence of parasporal crystals. From 391 *Bacillus*-like colonies observed through bright field and phase contrast microscopy, 28 isolates were identified as *Bt* based on the presence of crystalline inclusions. The isolates with crystalline inclusions were further purified as single colonies and stored as glycerol stocks at −80 °C for further studies. The shape of the crystalline inclusions varied among the *Bt* isolates. All the *Bt* isolates obtained from different soils of Western Ghats of Karnataka were Gram-positive (Supplementary data 3 and 4). On the basis of microscopic observations, parasporal crystals were classified into eight morphological classes; of frequency of occurrence: bipyramidal (28.57 % of the isolates) [11–13], irregular

(21.42 %), adhered to spore (14.28 %), rhomboidal (10.71 %), and cuboidal, small, rectangular, round shaped (3.57 %). Crystals were classified as "small" when they were not big enough to determine its shape at 1,000× magnification. About 36 % of the strains produced more than 1 crystal per sporangium. Almost half of the strains with bipyramidal crystals produced multiple crystals per cell, which only 18 % of the strains with round-shaped crystals produced multiple crystals per cell.

Molecular Analysis of Crystal Protein Profile in *Bt* Isolates

Isolates presented δ-endotoxins with molecular weights between 10 and 150 kDa, but the most common pattern was composed of proteins with molecular weights between 60–135 kDa (Fig. 1, 2, 3, 4). These strains commonly showed bipyramidal, irregular, adhered to spore, rhomboidal, cuboidal, small, rectangular, and round shaped forms of parasporal inclusions. Surprisingly, a ~55 kDa protein band was found to be present in most of the distinct profiles of isolates. Some of the isolates revealed the presence of 130–140 kDa bands of Cry proteins as reported by many authors (Supplementary data 4) [9, 11–14]. Few of the isolates showed an intense band of ~25 kDa. These unusual protein expression patterns, which may have resulted from proteolytic degradation [15]. Concurrent to report, many of the isolates in present study also showed a band of 44 kDa protein along with other bands. These results predicted that isolates obtained from Western Ghats of Karnataka contain possible novel Coleopteran-, Hemipteran- and Nematode-specific proteins. Based on the varied parasporal polypeptide patterns of the isolates, it can be concluded that *Bt* collection from this study has quite diversity of Cry proteins.

Determination of *cry* Type of Genes by PCR, Cloning, and Sequencing Analysis

In the present study, the personation and distribution of Coleopteran-specific and putative *cry* genes in Western gnats of Karnataka, India native *B. thuringiensis* isolates were studied. Characterization was based on PCR analysis using general and specific primers for all *cry* genes encoding proteins active against Coleoptera, Hempitera, and nematodes. These reports are useful for realizing the distribution of *cry* genes and may lead to the identification of effective isolates for application in biological control of pests, and novel candidate genes for bioengineered crop protection.PCR results showed that 28 native strains contained at least one Coleopteran, Hemipteran, and Nematode active *cry* gene. Strains containing *cry1I* and *cry9Da* genes were most abundant among Coleopteran-active *cry* genes

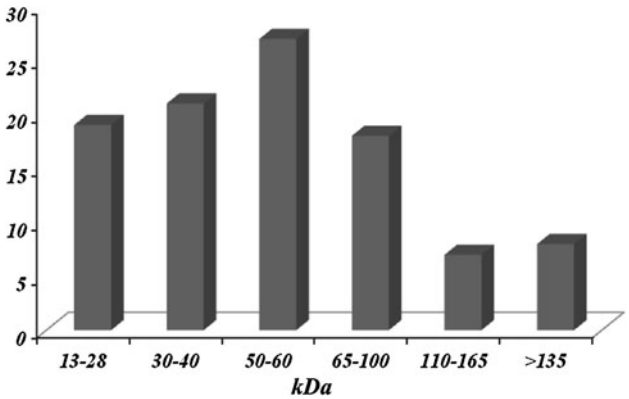

Fig. 1 Protein band patterns in field-collected strains of *B. thuringiensis* containing Coleopteran, Hemipteran and Nematode active *cry*-type genes identified by SDS–PAGE electrophoresis

and represented 64.28 and 21.42 % of the isolates, respectively. *Bt* strains harboring *cry6A* (39.28 %) gene were abundant among Nematode active *cry* genes where as *cry1Ab* (10.71 %) gene was found among Hemipteran active *cry* gene. Based on results of PCR utilizing general and gene specific primers, 13 distinct *cry* gene profiles were detected in this collection. Several of them were found to be different from all previously published profiles [16–18].

All the sequences were deposited at NCBI GenBank with accession numbers HQ018701, HQ018702, HQ018703, HQ018704, HQ018705, HQ018706, HQ018707, HQ018707, HQ018708, HQ018709, HQ018710, HQ018711, HQ018712, HQ018713, HQ018714, HQ018715, HQ018716, HQ018717, HQ018718, HQ018719 (*cry1I*), JF263124, JF263125 (*cry1*), JF263191 (*cry IIIC*), JF263044, JF263045, JF263046 (*cry7Ab*), JF263156, JF263157, JF263158, JF263159 (*cry8Ka*), JF26 3167, JK263168, JF263169, JF263170, JF263170, JF263171, JF263172 (*cry9Da*), JF263173 (*cry22*), JF263178, JF263179 (*cry28*), JF262999, JF263000, JF263001 (*cry5A*), JF263135, JF263136, JF263137, JF263138 (*cry5B*), JF263180, JF263181, JF263182, JF263183, JF263184, JF263185, JF263186, JF2631 87, JF263188, JF263189, JF263190 (*cry6A*), JF263098, JF2693099, JF26300 (*cry1Ab*), and JF263072, JF263073 (*cry4A*). In order to characterize novel *cry* sequences, PCR products were cloned to TA cloning vector pTZ57R/T [10] and sequenced. Comparisons showed 40–100 % homology with previously identified *cry* type genes.

Based on morphological and molecular studies, 28 isolates were selected for bioassays. A preliminary bioassay with highly concentrated spore–crystal suspensions of selected isolates was performed on first instar larvae of *Myllocerus undecimpustulatus undatus* Marshall. The selected strains showed different toxicity levels between 0 and 100 %. It is important to note that some isolates containing different *cry* genes profiles but showing the same

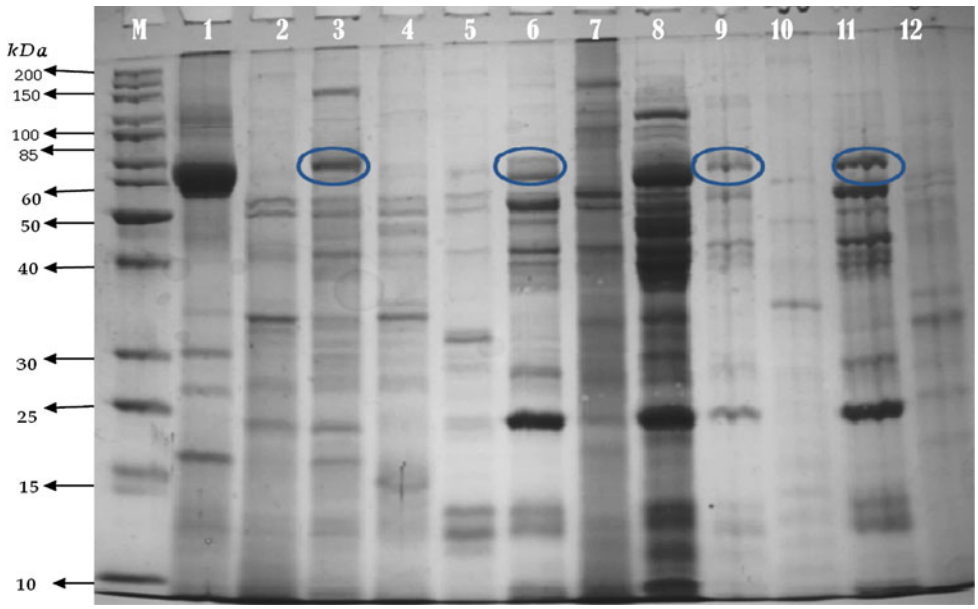

Fig. 2 Polyacrylamide gel electrophoretic pattern of total proteins of Sporulated *Bt*. The *lanes* from *left* to *right* show protein pattern of Karnataka isolates *Lane 1* KUN, *Lane 2* MAG, *Lane 3* BAJ, *Lane 4* ALG, *Lane 5* MAD, *Lane 6* SUB, *Lane 7* SHG, *Lane 8* UK, *Lane 9* CHM, *Lane 10* DHS, *Lane 11* MOD, and *Lane 12* GOR. *Blue circles* highlighting Coleopteran Protoxin (molecular mass-73 kDa) (Color figure online)

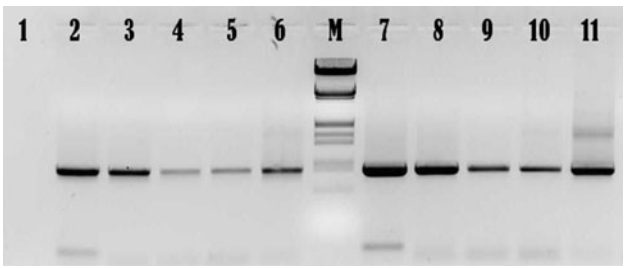

Fig. 3 Screening of putative isolates of *B.thuringiensis* from KARNATAKA using *cry9Da1* Coleopteran-active gene. Legend M-Molecular weight marker (*double digest*); *Lane 1* Genomic Negative, *Lane 2* GUN, *Lane 3* VEN, *Lane 4* DHA, *Lane 5* VIR, *Lane 6* UK, *Lane 7* UKK, *Lane 8* KAR, *Lane 9* KOP, *Lane 10* DUB, and *Lane 11* ARE

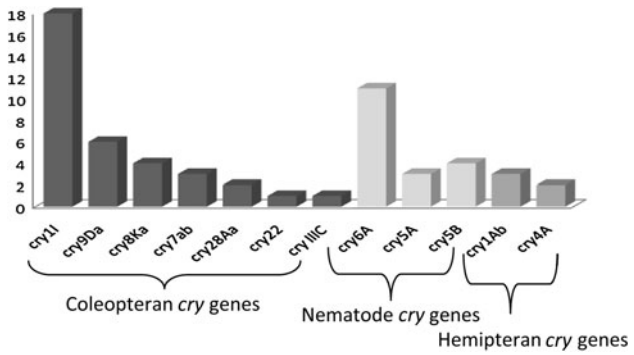

Fig. 4 Distribution of Coleopteran, Nematode and Hemipteran active *cry*-type genes obtained from 28 field-collected strains of *B. thuringiensis* identified by PCR analysis with universal and specific primers

toxicity against ash weevil larvae were identified, and also some of isolates with similar *cry* profiles showed different toxicity rate. However, three isolates showed more than 50 % mortality, and were further bioassayed with serial dilutions of spore–crystal mixtures for LC_{50} estimations (Table 1). According to the LC_{50} values and their fiducial limits, isolate DHA and VEN were the most toxic with 100 % toxicity in comparison with *B. thuringiensis* subsp. *morrisoni* pathovar *tenebrionis* as control with 80 % toxicity. Also isolates SAN showed 98 % toxicity. Although some *Bt* isolates were not toxic to the tested insects, these isolates can be active against other insects or cells.

Discussion

The characterization of *Bt* strains isolated from soil samples of Western Ghats of Karnataka, India presented here. This vast stretch of undisturbed land area has potential to support microbial diversity. This characterization contributes to an understanding of *Bt* molecular and genetic diversity, in the Western Ghats where such a study has not been conducted previously. *Bt* strains have been found worldwide from diverse habitats, including soil, insects, stored products, dust, deciduous and coniferous leaves, phyllospheres, and other miscellaneous habitats [12, 13, 19–22]. In the present study, the soil is an ideal source of *Bt*. A moderate-to-high frequency (76.0 %) of *Bt* isolates in the present study may be due to large amount of nutrients in the soil itself, allowing optimum survival and enrichment in the soils [23]. The *Bt* index value, which depends on the isolation procedures as well as the sampled materials, is an estimation of the success of a *Bt* isolation (Travers et al. [24]). The *Bt* index is also dependent on the type of substrate used for isolation [25, 26].

Table 1 Insecticidal activity of the selected *B. thuringiensis* isolates against *Myllocerus undecimpustulatus undatus* Marshall (Coleoptera: Curculionidae) larvae

Isolate	LC50[a] (ng/cm^2)	Confidence limit (lower and upper) (95%)[b]	Mortality (%)[c]
DHA	–	–	100[d]
SAN	165	148–183	98
VEN	–	–	100[d]
Bt. subsp. *morrisoni*	178	154–193	80

[a] Results are expressed as nanograms of toxin per square centimeter of surface

[b] Fl95 min.–max., 95% confidence limit

[c] The highest mortality (%) observed in the bioassays (at 300 ng toxin/cm^2)

[d] LC50 value has not been obtained, the stated amount causes 100% mortality

In general, Cry proteins are active against Lepidopteran (Cry1 of 130–140 kDa), both Lepidopteran and Diptera (Cry II of 71 kDa), Coleopteran (Cry III of 66–77 kDa) and Diptera (Cry IV of 125–145 and 68 kDa) larvae [14, 27, 28]. In our studies, characteristically, isolates possessed δ-endotoxins with molecular weights between 10 and 150 kDa, among which 130, 55, 34, and 25 kDa were distinctly present signifying their spectrum of activity against Coleopteran, Hemipteran, and Nematode pests. The large-sized proteins of 130 kDa are also characteristic of numerous anti-Lepidopteran toxins of Cry1 category, example Cry1A [8], Cry1B [29, 30], Cry1C [11], Cry 1F [31], Cry1I [32], or Cry 1K [33]. Cry 3 and Cry5 shows toxicity against Coleopteran or Coleopteran and Lepidopteran larvae [16, 19, 33]. It is well known that *Bt* produces endogenous proteases and that their production may vary considerably among strains [26, 34]. A few crystalline inclusions were composed of the small components of polypeptides of 23.4–45 kDa. The size of these proteins may indicate anti-Coleopteran δ-endotoxins of Cry3 and Cry35 subclasses (39–55 kDa) [2, 7, 11, 35–38].

It could be explained by the possibility of production Cry proteins encoded by unknown genes. Moreover, some *cry* genes could have low expression or even be inactive [16]. In addition, some Cry proteins do not participate in creating inclusion. Toxins, for example Cry1I, can also be secreted into the medium [15]. Difficulties in estimating the Cry1 composition in the SDS-PAGE preparation could be caused by the presence of protoxins, toxins, and proteolysis intermediates in a sample [39]. Moreover, problems with determining the mass of Cry toxins could be due to a more compact structure of proteins profiles than the protein standard [6].

The identification of known *cry* genes in *Bt* strains is important, since the specificity of action is known for many of the Cry toxins. This fact allows the possibility of selecting native strains that could be used in control of some targets and of selecting strains with the highest activity. PCR is a tool that has been widely used for characterization of genes coding for Cry proteins and for analysis of *Bt* collections [3, 5, 15, 21, 37, 40–42]. PCR technique was first introduced by to identify *cry* genes to predict insecticidal activity [43]. Over the last decade, PCR methods for screening *cry* genes present in *Bt* collections, including multiplex PCR methods, have been used to identify strains that harbor genes coding for known Cry types [3, 5, 21, 22, 39].Out of 185 strains in the collections are potentially active against Lepidoptera using *cry1, cry2*, and *cry9A* primers; 76 strains were potentially active against Diptera using *cry4, cry10, cry11, cry21A*, and *cry27Aa* primers; 8 strains may be toxic to Coleoptera using *cry7* and *cry8* primers; and 3 strains may be Nematicidal using *cry5, cry12*, and *cry14* primers [27]. Two strains namely SN1 and KH4 containing Coleopteran-active *cry* genes showed higher activity against *X. luteola* larvae at 152 and 165 ng/cm^2, respectively, than *B. thuringiensis* subsp. *morrisoni* pathovar *tenebrionis* 170 ng/cm^2 [44].Standardization assays often involve the use of purified or extracted crystal proteins (Cry and Cyt toxins) and do not include any of the other toxins and synergists (e.g., chitinases) produced by these species of bacteria [45]. Continuous sub-culturing of *Bt* can be problematic because it may cause a decline in toxicity [17]. In conclusions, these strains are candidates for harboring novel *cry* gene sequences. The bioassay of the selected isolates against other pests, characterization of the observed potentially novel *cry* genes and the search for additional novel genes will be continued. Thus, future investigation of this study will include bioassays against resistant insects and various other insects to explore their toxic potential and cloning of novel *cry* genes. These new isolates may be used as potential entomopathogens in biological control and applied to integrated pest management of agriculturally important insect pest.

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