Journal of Biological Control, 27(4): 298–303, 2013

Research Article

Molecular characterization of *Bacillus* isolates with insecticidal activity against greater wax moth, *Galleria mellonella* L. (Pyralidae: Lepidoptera)

NAYIMABANU TAREDAHALLI*, N. S. BHAT[1], A. R. V. KUMAR and M. S. JAKHAR[2]

Department of Entomology, University of Agricultural Sciences, Gandhi Krishi Vignana Kendra (GKVK), Bangalore 560 065, Karnataka, India

[1]Department of Apiculture, University of Agricultural Sciences, GKVK, Bangalore 560 065.

[2]Assistant Director, Bureau of Indian Standards, Parwanoo, Solan 173 220

**Corresponding author E-mail* : aditims66@gmail.com

ABSTRACT: Six local *Bacillus* strains isolated from soils from different parts of Karnataka were characterized by their high toxicity against third instar larvae of greater wax moth (*Galleria mellonella* L.). The PCR amplification analysis using *B. thuringiensis* specific primers revealed that only four isolates are *Bt* isolates. The PCR amplification analysis for *cry* genes indicated the presence of amplified fragments characteristic of three different *cry* genes *cry*1Aa, c*ry*2Aa and *cry*11Aa. Results showed the presence of *cry11Aa* and *cry1Aa* in isolate N12 with expected sizes of 816bp and 891-1089bp, respectively. *Bt* isolate; A-7 harbored *cry1Aa, cry2Aa* and *cry11Aa* with product size 891-1308, 235-696 and 636bp, respectively. While the isolates M-2 and A3 contained only one gene *cry2Aa* with a product size 696-735bp. The two isolates; N-12 and A-7 were harbored multiple *cry* genes. The *cry2Aa* genes were the most frequently found among the three local *Bt* isolates. The profiles of *cry* genes indicated that all isolates were active against lepidopteran insects, and therefore these strains could be potential candidates in the search for biocontrol agents in management of greater wax moth, *G. mellonella* with specific lepidopteran activity.

KEY WORDS: *Galleria mellonella, Bacillus thuringiensis,* PCR, *cry* genes, Lepidoptera

(Article chronicle: Received: 10-08-2013; Revised: 11-10-2013; Accepted: 15-11-2013)

INTRODUCTION

The greater wax moth is responsible for heavy economic losses reaching up to 60–70 per cent to bee keepers in India (Paddock, 1918, Kapil and Sihag, 1983; Hanumanthaswamy *et al.,* 2009). Of all the various microbial agents that have been evaluated, the most successful by far, has been the *Bacillus thuringiensis* Berliner. It does not affect Hymenoptera and is considered nonpathogenic to human (Gates, 1986).

Bacillus thuringiensis is a gram-positive, spore-forming bacterium with entomopathogenic properties and produces insecticidal proteins during the sporulation phase as parasporal crystals. These toxins are highly specific to their target insect and are generally expected to be innocuous to humans, vertebrates and plants, and are completely biodegradable (Bravo *et al*., 2005). A few attempts that were made in India to isolate this bacterium have yielded cultures that are effective against *Plutella xylostella,* (Karthik John, 2001), *Helicoverpa armigera* (Reddy, 2000); *Myllocerus subfasciatus* (Raghunatha, 2002); and *Bactrocera cucurbitae* and *Musca domestica* (Smitha, 2009). There are reports on the isolates effective against *Galleria mellonella* L. In our previous study (Taredahalli *et al*., 2011), we have isolated 98 *Bacillus* isolates and characterized morphologically and biochemically and found six *Bacillus* isolates with good insecticidal activity against *G. mellonella* L. Therefore, the present study was conducted to characterize the *Bacillus* isolates for their identity and presence of *cry* genes with a view to identify strains that could be deployed for the management of *G. mellonella* under field conditions.

MATERIALS AND METHODS

Bacterial Strains

Six local *B. thuringiensis* isolate strains (C7, N12, F2, A3, A7, and M2) were used in the study. They were previously isolated from the soil samples collected from different parts of Karnataka and characterized by their high toxicity against third instar larvae of *G. mellonella* (Fig. 1).

Isolation of Bacterial Genomic DNA

Isolation of genomic DNA was carried out by the methods of Sambrook and Russel (2001). *Bacillus thuringiensis* strains were grown in 10 ml of LB broth at 30°C with shaking overnight. Cells were harvested by centrifuging at 6000 rpm for 10 min and the cell pellet was resuspended in 567 TE buffer vertexed. Then 30ìl lysozyme/ml, 30 ìl SDS, 3 ìl protienase K was added and mixed well followed by the incubation for 1 hour at 37°C. 100 ìl 5 M NaCl and 80 ìl CTAB/NaCl solution were then added and mixed by inverting the tubes. The cell suspension was incubated for 10 min at 68°C in a water bath. For chloroform extraction, one volume of chloroform/isoamyl alcohol

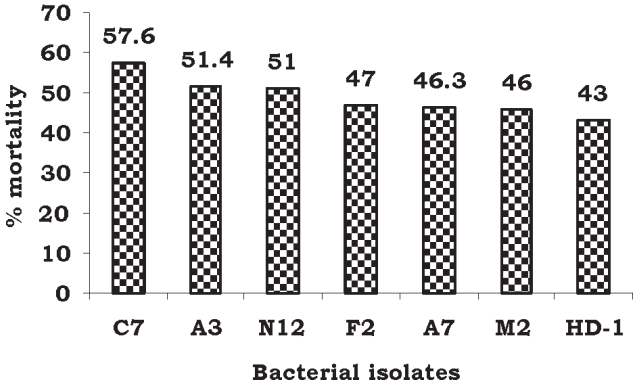

Fig. 1. Mortality of third instar larvae of *Galleria mellonella* on exposure to various *Bacillus* isolates

(24:1) was added and mixed effectively and centrifuged at 10000 rpm for 10 min. Then aqueous phase was phenol:chloroform:isoamyl alcohol (25:24:1) was added and centrifuged at 10000 rpm for 10 min. The aqueous phase was transferred into another tube and 0.5 M NaCl final concentration and two volumes of cold absolute ethanol were added to precipitate the DNA and kept at –20°C for 1 hour. After spinning at 10,000 rpm for 10 min, pellets were washed in 500 ìl 70% ethanol by centrifugation for 5 min at 8,000 rpm. Finally, the washed DNA pellets were dried and resuspended in 50-200 ìl 1X TE, depending on the amount of the DNA pellet. Samples were stored at –20°C until further use.

Identification of *Bt* strains using species specific primers

PCR assays were performed using PCR primer sets designed from the gyrase subunit B gene (*gyr*B) to differentiate *B. thuringiensis* (BT1 and BT2r) from other *Bacillus* species (Yamada *et al*., 1999). DNA amplification was carried out in a reaction mixture containing 2 µl bacterial DNA, 4 µL of 1 mM dNTP, 20 pmol of appropriate primer pairs for *B. thuringiensis* (BT1 [5'-ATCGG TGATACA GATAAGACT-3', positions 175 to 195] and BT2r [5'-CCTTCATACGTATGAATATTATTT-3', positions 519 to 542]), 2.4 µL of 25 mM MgCl2, 1 U/µl Taq polymerase, and sterile distilled water to the total volume of 20 µL. Amplification reagents were obtained from Thermo scientific (Lithuania). The primers were purchased from Sigma-Adrich (Bangalore). Each experiment was conducted with a negative control (without template DNA). Primer sequences and conditions of PCR amplification for *cry* gene identification were accomplished as described by Beron *et al.* (2005). The PCR products were electrophoresed in 0.8% agarose gel and stained with ethidium bromide. The results were documented by the Easywin32 system (HeroLab, Germany). The molecular weights of amplified products were estimated by comparing with molecular weight marker (Fermentas).

Identification of *cry* genes by PCR

Five degenerate primers were used for the detection of novel *cry* genes from *B. thuringiensis* strains (Beron *et al.,* 2005). DNA amplification was carried out in a reaction mixture containing 2 µl bacterial DNA, 4 µL of 1 mM dNTP, 20 pmol of appropriate primer pairs (Table 1), 2.4 µL of 25 mM MgCl2, 1 U/µl Taq polymerase, and sterile distilled water to the total volume of 20 µL. Amplification reagents were obtained from Thermo scientific (Lithuania). The primers were purchased from Sigma (Bangalore). Each experiment was conducted with a negative control (without template DNA). Primer sequences and conditions of PCR amplification for *cry* gene identification were accomplished as described by Beron *et al.* (2005). The PCR products were electrophoresed in 0.8% agarose gel and stained with ethidium bromide. The results were documented by the Easywin32 system (HeroLab, Germany). The molecular weights of amplified products were estimated by comparing with molecular weight marker (Fermentas).

RESULTS AND DISCUSSION

Out of six isolates analyzed, four namely N12, M2, A7 and A3 were amplified with the product size of 450bp indicating the characteristic of *B. thuringiensis*. The standard strain *Bt. kurstuki* (HD-1) was also amplified with same product size of 450bp. There was no amplification observed in two isolates C7 and F2 which revealed that these two are non-*B. thuringiensis* isolates. This is one of the important techniques used for differentiation of *Bacillus* species. Sequencing of the *gyrA* and *rpoB* genes has been found to be useful in discriminating species of the *B. subtilis* group, while the *gyrB* gene has been studied

Table 1. Characteristics of the general primers used and predicted sizes of DNA PCR amplification fragments of known *cry* genes

Primerpair	Primer sequence (5'-3')	Predicted positions	Expected product size (bp)								
			cry1Aa	*cry3Aa*	*cry4Aa*	*cry7Aa*	*cry8Aa*	*cry9Aa*	*cry2Aa*	*cry11Aa*	*cry14Aa*
OL1 (d)OL5 (r)	5'TAHCANYATAYGCACARGCHGCMAAYTTH CAT 5' GGAATAAATTCRATTYTRTCTATATAAA	510-1788	1308	1311	1374	1317	1362	1359	735	816	1377
OL1 (d)OL4 (r)	5'TAHCANYATAYGCACARGCHGCMAAYTTH CAT5' AGCATADCGRAHNCYHRYDYVATA	510-1566	1,089	1122	1125	1080	1137	1134	696	771	1113
OL2 (d)OL5 (r)	5'AGAGAHRTGAHWDTDAHRGTATTRGAT5' GGAATAAATTCRATTYTRTCTATATAAA	699-1788	1119	1122	1143	1128	1167	1158	558	636	1152
OL2 (d)OL4 (r)	5'AGAGAHRTGAHWDTDAHRGTATTRGAT5' AGCATADCGRAHNCYHRYDYVATA	699-1566	891	900	894	891	942	933	519	591	888
OL3 (d)OL5 (r)	5' TATBRHRYDRGNDTYCGHTATGCT5' GGAA TAAATTCRATTYTRTCTATATAAA	1566-1788	252	246	273	261	249	249	**57**	**54**	**288**

The primer are degenerate primers, and the sequences are indicated according to the degenerate DNA genetic code as follows: B=C, G, or T; D=A, G, or T; H=A, C, or T; M=A or C; R=A or G; Y=T or C; W=A or T; N=A, C, G, or T; and V=A, C, or G.

(Bero´n *et al*., 2005)

Table 2. PCR analysis for the presence of *cry* genes in different *Bacillus thuringiensis* isolates

Sl. No.	Primer pair	*Cry* gene detected									
		N12		A7		M2		A3		HD1	
		cry gene	Product size (bp)	*cry gene*	Product size (bp)	*cry gene*	Product size (bp)	*cry gene*	Product size (bp)	*cry gene*	Product size (bp)
1.	OL1(d) OL5(r)	*cry11Aa*	816	*cry1Aa* *cry2Aa*	1308 235	*cry2Aa*	735	*cry2Aa*	735	*cry2Aa*	735
2.	OL1(d) OL4(r)	*cry1Aa*	1089	*cry1Aa* *cry2Aa*	1089 696	*cry2Aa*	696	– –		*cry2Aa*	696
3.	OL2(d) OL5(r)	–		*cry1Aa* *cry11Aa*	1119 636	– –		– –		*cry1Aa*	1119
4.	OL2(d) OL4(r)	*cry1Aa*	891	*cry1Aa*	891	–		–		*cry1Aa*	891
5.	OL3(d) OL5(r)	–	–	–		–		–		–	

for the discrimination of members of the *Bacillus cereus* group (Chun and Bae, 2000; Palmisano *et al*., 2001). Suitable primer sets have been reported that could amplify the *gyrB* fragment of *B. thuringiensis*, *B. anthracis, B. mycoides* and *B. cereus* to specifically identify the organism irrespective of their phenotypes, serotypes and virulence factors (Yamada *et al*., 1999). The same gene was also used to differentiate members of *Bacillus* or related endospore-forming genera in contaminants in semifinal gelatin extracts by Clerk *et al*. (2004). Therefore, in the present study also the same technique has been used successfully.

Identification of *cry* genes by PCR

The *cry* gene amplification analysis of the four *Bacillus* isolates which were proved to be *B. thuringiensis* was carried out using 5 sets of degenerated primers (Table 1). The results revealed the presence of amplified fragments characteristic of three different *cry* genes *viz.cry1Aa*, *cry11Aa* and *cry2Aa* with the expected sizes of PCR products of ranging from 891 to 1308, 636 to 816 and 235 to 735 bp, respectively (Fig. 3 and Table 2). Excepting isolate N12, *cry 2Aa* gene was common in other three *B. thuringiensis* isolates including standard HD-1 (Table 2). All four *B. thuringiensis* isolates were harboring a combination of different lepidoptera-active *cry* genes, such as N12 harbored *cry1Aa* and *cry11Aa* with PCR product size 891-1089 and 816bp, respectively; A-7 harbored *cry1Aa, cry 2Aa* and *cry11Aa* with product size 891-1308, 235-696 and 636bp, respectively, while M-2 and A-3 were containing a single gene that is *cry2Aa* of size 696-735bp (Fig. 3 and Table 3). Since proteins of groups cry1, cry2 are toxic for pests of the order Lepidoptera (e.g., *Lymantria dispar* [Peyronmet, *et al*., 1998], *Manduca sexta* [Zeigler, 1999], *Spodoptera littoralis* [Kumar *et al*., 1998], and *Helicoverpa armigera* [Jalali *et al*., 2004]), these strains can be potential candidates

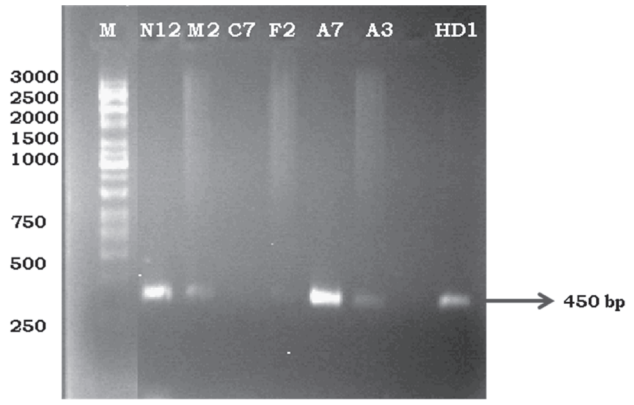

Fig. 2. PCR products using the *Bt* specific primers for different *Bacillus* isolates. M.1Kb marker

in the search for biocontrol agents in management of *G. mellonella* with a specific lepidopteran activity. The profiles of *cry* genes indicated that all isolates were active against lepidopteran insects. The two isolates; N-12 and A-7 are harboring multiple *cry* genes (Fig. 3). The presence of different *cry* genes in the same *B. thuringiensis* strain has been reported, for example Aronson (1994) and Ben-Dov *et al.* (1997) reported the presence of *cry1* genes and *cry3*, *cry8*, or *cry7* genes in the same *B. thuringiensis* strain. While, Bravo *et al.* (1998) observed lepidopteran-active *cry1* genes and coleopteran-active *cry3A*, *cry3Ba* and *cry7A* genes in the same strain. They suggested that such *B. thuringiensis* strains which harbored more than one *cry* gene, have a high frequency of genetic information exchange.

The *cry 2Aa* genes were the most frequently found among the 3 local *B. thuringiensis* isolates and the second abundant gene was *cry1Aa* (Table 2). Ben-Dov *et al.* (1997) found that strains containing *cry1* genes were the most abundant; however, strains harboring *cry4* genes were the second most abundant, while strains with *cry3* genes were absent. While, Chak *et al*. (1994) reported that *cry1A* genes were the most abundant, followed by the *cry1C* and *cry1D* genes. The identification of known *cry* genes in *B. thuringiensis* 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 the control of some targets and of selecting strains with the highest activity.

The PCR screening is a rapid method for detecting and differentiating *B. thuringiensis* field strains by their PCR product profiles and for predicting their insecticidal activities in order to direct them for subsequent toxicity assays against lepidoptera, coleoptera and diptera. The two *B. thuringiensis* isolates displaying multiple profiles that contain combinations of *cry* genes should be characterized and further developed for integration with other toxicity assays against other lepidopteran insects. Specific gene identification using specific set of primers could be carried out for further analysis of *cry* gene specific to the target insect Ben-Dov *et al.* (1997).

ACKNOWLEDGEMENTS

Authors are grateful to Ms. Kumari Manju, Dr. B. Ravi Kumar, and Mr. K.V. Prakash for their constructive help and support at various stages of the study. Dr. R. Rangeshwaran, NBAII, Bangalore provided the standard HD1 culture. The work was carried out at the molecular lab of the Department of Entomology, University of Agricultural Sciences, GKVK, Bangalore, developed

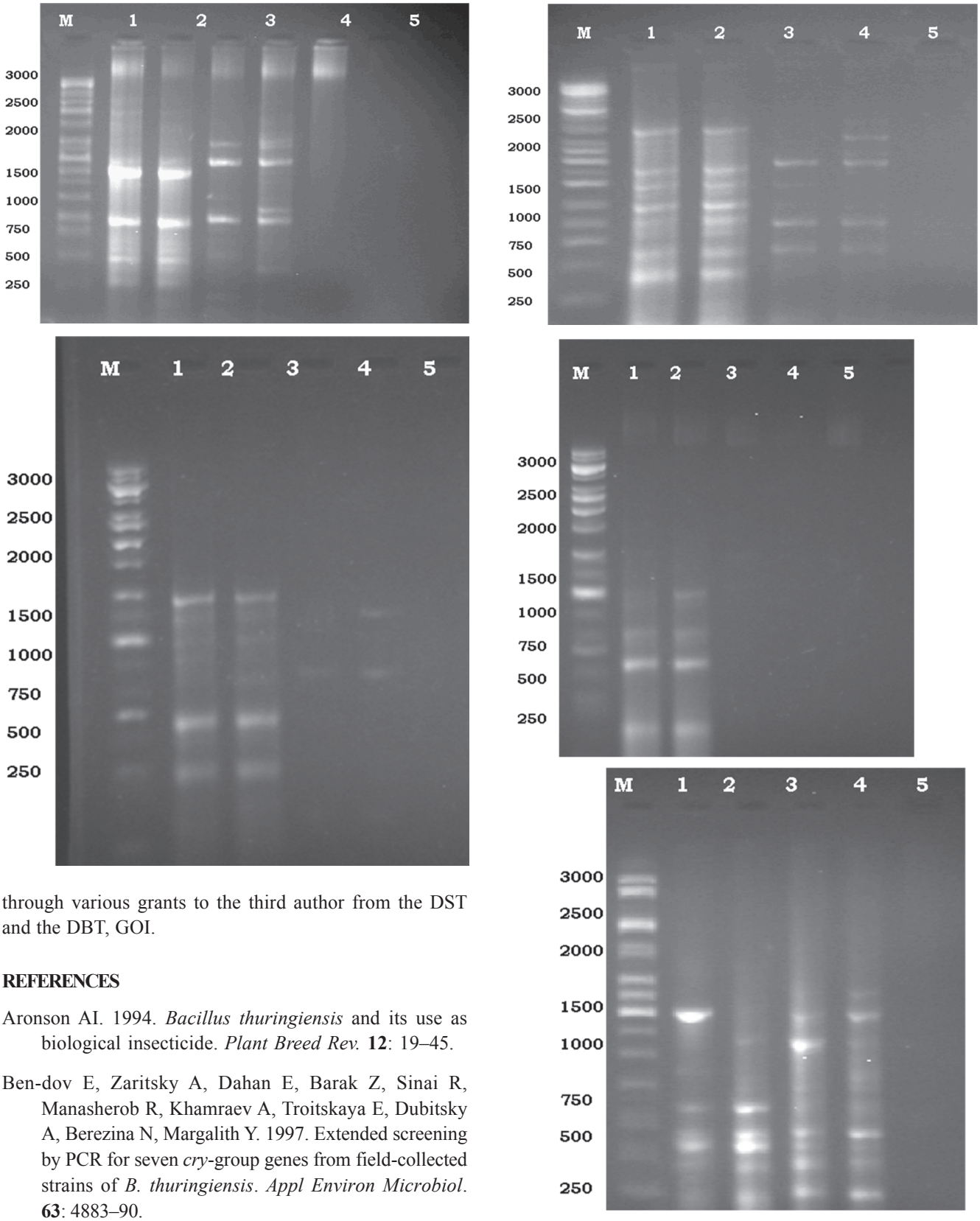

Fig. 3. PCR products using the primers for novel *cry* genes for isolates (A). N12, (B). A7, (C). M2, (D). A3, (E). HD 1. Legend: Lanes 1, 2, 3, 4 and 5 represents primer pair 1, 2, 3, 4 and 5 respectively for respective isolates. M.1Kb marker

through various grants to the third author from the DST and the DBT, GOI.

REFERENCES

Aronson AI. 1994. *Bacillus thuringiensis* and its use as biological insecticide. *Plant Breed Rev.* **12**: 19–45.

Ben-dov E, Zaritsky A, Dahan E, Barak Z, Sinai R, Manasherob R, Khamraev A, Troitskaya E, Dubitsky A, Berezina N, Margalith Y. 1997. Extended screening by PCR for seven *cry*-group genes from field-collected strains of *B. thuringiensis*. *Appl Environ Microbiol*. **63**: 4883–90.

Beron CM, Curatti L, Salerno GL. 2005. New strategy for identification of novel *cry*-type genes from *Bacillus thuringiensis* strains. *Appl Environ Microbiol.* **71**: 761–765.

Bravo A, Sarabia S, Lopez L, Ontiveros H, Abarca C, Ortiz A, Soberon M, Quintero R. 1998. Characterization of *cry* genes in a Mexican *Bacillus thuringiensis* strain collection. *Appl Environ Microbiol*. **64**: 4965–4972.

Chak KF, Chao DC, Tseng MY, Kao SS, Tuan SJ, Feng TY. 1994. Determination and distribution of *cry*-type genes of *Bacillus thuringiensis* isolates from Taiwan. *Appl Environ Microbiol*. **60**: 2415–2420.

Gates NJ. 1986. Wax moth control. *Glean. Bee culture*. **144**: 407.

Hanumanthaswamy BC. 2000. Natural enemies of honey bees with special reference to bio ecology and management of greater wax moth, *Galleria mellonella* L (Lepidoptera: Pyralidae). Ph.D. Thesis, UAS, Bangalore, India.

Jalali SK, Mohan KS, Singh SP, Manjunath TM, Lalitha Y. 2004. Baseline-susceptibility of the old-world bollworm, *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae) populations from India to *Bacillus thuringiensis Cry1Ac* insecticidal protein. *Crop Protect*. **23**: 53–59.

Kapil RP, Sihag RC. 1983. Wax moth and its control. *Indian Bee J.* **45**: 47–49.

Karthik J. 2001. Evaluation and molecular characterization of *Bacillus thuringiensis* for the control of diamond-back moth (*Plutella xylostella*). M.Sc. Thesis. 88 pp.

Kumar NS, Venkateswerlu G. 1998. Endogenous protease-activated 66-kDa toxin from *Bacillus thuringiensis* subsp. *kurstaki* active against *Spodoptera littoralis. FEMS Microbiol Lett.* **159**: 113–120.

Paddock FB. 1918. The bee moth or wax worm. *Texas Agri, Expt Station Bull*. **2**: 231.

Peyronmet O, Vachon V, Schwartz JL, Laprade R. 2001. Ion channels induced in planar lipid bilayers by the *Bacillus thuringiensis* toxin *Cry1Aa* in the presence of gypsy moth (*Lymantria dispar*) brush border membrane. *J Membr Biol.* **184**: 45–54.

Raghunatha R. 2002. Evaluation of local isolates of *Bacillus thuringiensis* Berliner against the brinjal ash weevil *Myllocerrus subfasciatus* Guerin. (Coleoptera: Curculionidae). M.Sc. Thesis. 67 pp.

Reddy DCL. 2000. Isolation and molecular characterization of *Bacillus thuringiensis* for lepidopteran insect control. M.Sc. Thesis. 83 pp.

Sambrook W, Russel DW. 2001. *Molecular cloning: a laboratory manual*. 3rd Ed. New York: Gold Spring Harbor Laboratory. 173 pp.

Smitha G. 2009. Isolation, characterization and evaluation of diptera (Hexapoda: Insecta) specific *Bacillus thuringiensis* Berliner from different soils. M.Sc. Thesis. 129 pp.

Taredhalli N, Bhat NS, Kumar ARV. 2011. Diversity of *Bacillus* spp. in soil environment and their toxicity against greater wax moth *Galleria mellonella* L. pp. 167–172. In: Dunston P. Ambrose, *Insect Pest Management, A Current Scenario, 2011* (ed.), Entomology Research Unit, St. Xavier's College, Palayamkottai, India.

Uribe D, MartineZ W, Ceron J. 2003. Distribution and diversity of *cry* genes in native strains of *Bacillus thuringiensis* obtained from different ecosystems from Columbia. *J Inv Pathol*. **82**: 119–127.

Yamada S, Ohashi E, Agata N, Venkateswaran K. 1999. Cloning and nucleotide sequence analysis of *gyr* B of *Bacillus cereus*, *B. thuringiensis*, *B. mycoides*, and *B. anthracis* and their application to the detection of *B. cereus* in rice. *Appl Environ Microbiol*. **65**: 1483–1490.

Zeigler DR. 1999. *Bacillus thuringiensis* and *Bacillus cereus*. In *Bacillus genetic stock center catalog of strains*, Vol. 2, 7th edn, The Ohio State University, Columbus, Ohio. Available at: http:// www.bgoc.org/ catalogs/catpart2.pdf