ENTOMON 35(4): 1–8 (2010)
Article No. ent.35401

Bioefficacy of native *Bacillus thuringiensis* isolates from the Western Ghats of Kerala on pumpkin caterpillar, *Diaphania indica* (Saund.) (Lepidoptera: Pyralidae)

P. M. Neema, D. Girija* and M. P. Mathew

Centre for Plant Biotechnology and Molecular Biology, Kerala Agricultural University, Thrissur 680 656, Kerala, India
Email: devakigirija@gmail.com

ABSTRACT: Bioefficacy of crude protein from eleven *Bacillus thuringiensis* isolates obtained from the Western Ghats region of Kerala was tested by diet contamination method, on second instar larvae of pumpkin caterpillar, *Diaphania indica* (Saund), a major lepidopteran pest of cucurbitaceous vegetables in Kerala. Based on statistical analysis of per cent mortality of larvae, the isolates KK7, KK8, KK9 and KY2 were on par, but significantly less efficient than the reference strain HD1. Profiling of *cry*1 and *cry*4 genes from *B. thuringiensis* isolates was done using universal *cry*1 and *cry*4 primers for detection of *cry* genes and prediction of their insecticidal activities. Amplification with universal *cry*1 gene primer was obtained for eight isolates KY2, KY3, KY5, KY6, KK7, KY8, KY9 and EM11 along with HD1. Amplification for *cry*4 gene was obtained for isolates KY1 and EM10 but not *cry*1 gene. KY4 did not show *cry*1 or *cry*4 gene though it was also toxic to *D. indica*. New genes, unidentified in the present investigation, are involved in the toxicity of isolates without *cry*1 gene.
© 2010 Association for Advancement of Entomology

KEYWORDS: *Bacillus thuringiensis*, *Diaphania indica*, bioefficacy, crystal proteins, *cry* gene

INTRODUCTION

The crystalliferous bacterium, *Bacillus thuringiensis* Berliner is the most widely used environment friendly alternative to chemical pesticides for the biological control of pests of agricultural crops and forest trees (Lambert, 1992). Research efforts to improve formulations based on the bacterium to increase the toxicity spectrum or to understand the mechanism of action of toxins produced by the bacilli heavily rely on bioassay against target pests. The development of transgenic plants expressing the

*Corresponding author

© 2010 Association for Advancement of Entomology

B. thuringiensis toxin and their significant use in integrated pest management created interest for new isolates of this micro organism against lepidopteran pests. Moreover, there is an increasing concern over development of resistance when a single gene is used in transgenics. Western Ghats is one among the 18 biodiversity hotspots in the World and is least explored for microbial diversity. The present investigations were directed at exploring the bioefficacy of native *B. thuringiensis* isolates from the Western Ghats of Kerala against a serious lepidopteran pest of cucurbitaceous vegetables, the pumpkin caterpillar, *Diaphania indica* (Saund.).

MATERIALS AND METHODS

Laboratory experiments were conducted to assess the bioefficacy of *B. thuringiensis* isolates against *D. indica*. Eleven bacterial isolates were obtained from soils of the Western Ghats from the districts of Kottayam, Kozhikode and Ernakulam, by Travers' method (Travers *et al.*, 1987). These isolates along with the reference strain HD1 were stored as stabs in cryostorage vials in LB agar medium under refrigerated condition.

Crude preparation of crystal protein was extracted from *B. thuringiensis* (Dulmage, 1970). The toxicity of crystal protein was tested on four- to five day old larvae of *D. indica* by diet contamination method, as described by Schesser *et al.* (1977).

Author: Check year '1977' or '1997'

Larval mortality was recorded at an interval of 24 h, till all the larvae died/pupated and the per cent mortality was corrected using Abbot's formula.

Statistical analysis was done using Kendall's coefficient of concordance by SPSS software, a statistical package (Kendall and Smith, 1939). The isolates were ranked on the basis of mortality on fifth and eighth days.

Screening of isolates for the presence of *cry*1 and *cry*4 genes was done using universal primers designed by Ben Dov *et al.* (1997). The total DNA of *B. thuringiensis* isolates and reference strains used for PCR amplification was isolated following the procedure of Sambrook and Russel (2001). A set of universal primers Un1(d), Un1(r), Un4(d) and Un4(r) (Ben Dov *et al.*, 1997) were used for the amplification of *cry*1 and *cry*4 genes. The primers were custom synthesized by Integrated DNA Technologies, USA. Other components of PCR reaction mix were obtained from Bangalore Genei. PCR was carried out by standard procedure (Sambrook and Russel, 2001) in Eppendorf Master Cycler, Gradient (Eppendorf, Germany) under the following conditions: 2 min of initial denaturation at 94 °C, followed by 30 cycles of denaturation for 45 seconds at 94 °C, annealing for 1 min at 55 °C depending on DNA template and extension for 2 min at 72 °C. A final extension of 10 min at 72 °C was given after completion of 35 cycles. PCR products were resolved on 0.8 per cent agarose gel stained with ethidium bromide and λ DNA/*Eco* R1+ *Hind* III double digest as a DNA molecular weight marker. The DNA bands in gels were documented using gel documentation system (Alpha imager TM1200).

Author: Please check name 'Ben-Dov' changed to 'Ben Dov' in text

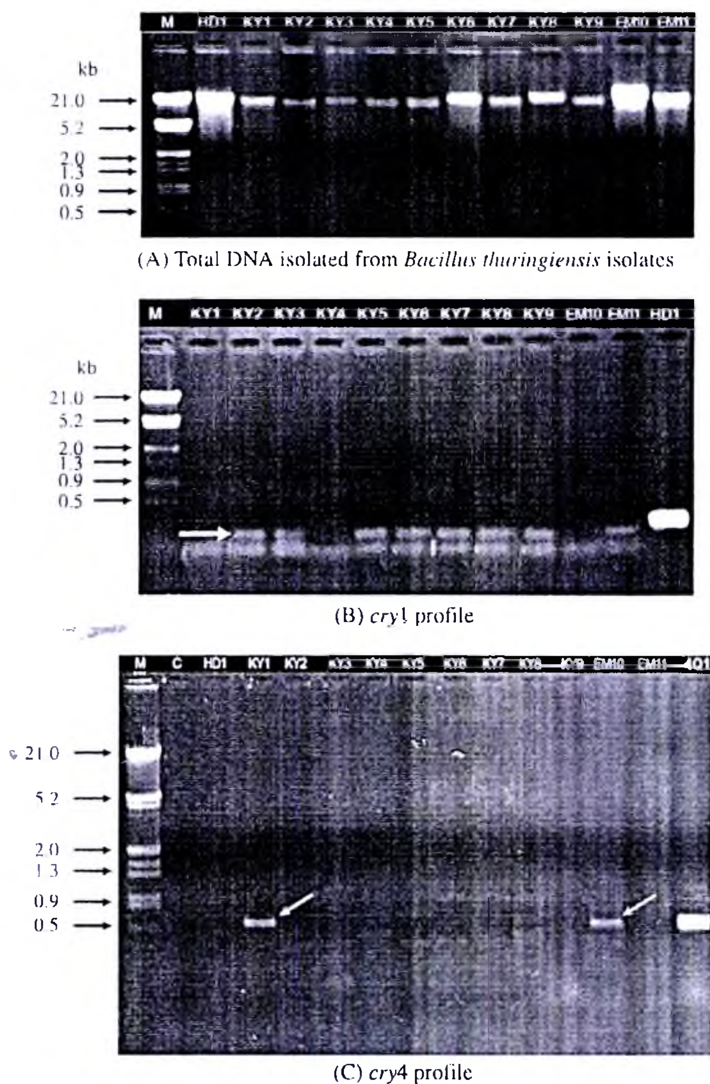
TABLE 1. Mortality and mean rank score of crystal proteins of *Bacillus thuringiensis* isolates on *Diaphania indica* larvae

Isolate	5th day		8th day	
	Per cent Mortality	Mean Rank Score	Per cent mortality	Mean Rank Score
KY1	57.7	6.13	94.4	4.64
KY2	70.1	8.50	84.4	7.57
KY3	65.1	2.88	100	5.21
KY4	70.1	1.50	94.4	3.57
KY5	70.1	7.00	100	6.36
KY6	62.6	6.38	94.4	6.00
KK7	85	8.75	95	9.14
KK8	85	8.50	100	9.29
KK9	70.2	8.50	89.2	8.14
EM10	50.4	6.00	88.8	4.34
EM11	38.3	2.13	76.1	1.64
HD1	100	11.75	100	12.50

RESULTS

The mortality response of *D. indica* larvae to *B. thuringiensis* crystal proteins revealed that the reference strain HD1 caused 100% mortality by fifth day. Among the native isolates, highest mortality was obtained for the isolate KK7 and KK8, with 85% mortality on fifth day and 85% and 100% mortality on 8th day. This was followed by the isolates KK9, KY2, KY4 and KY5 with about 70% mortality on 5th day and 84–100% mortality on 8th day (Table 1). HD1 showed the highest rank scores (11.75 and 12.50 on 5th and 8th days, respectively). Isolate KK7 proved to be a potential isolate with a mean score of 8.75. Isolates KK8, KK9 and KY2 had the same efficacy with a mean rank score of 8.5.

Amplification was obtained for *cry*1 gene primer, at an annealing temperature of 55 °C. Amplification of a single band of less than 250 bp was obtained with universal *cry*1 gene primer for eight isolates KY2, KY3, KY5, KY6, KK7 KK8, KK9 and EM11 and strain HD1 (Fig. 1, Table 2). No amplification was obtained for isolates KY1, KY4 and EM10. Screening of *B. thuringiensis* isolates was carried out for *cry*4 gene with universal *cry*4 primer (Ben Dov *et al.*, 1997). Amplification for *cry*4 gene was obtained for reference strain 4Q1 and native isolates KY1 and EM10. Single band of 575 bp was obtained for isolate KY1 and three bands of 531, 942 and 1245 bp for EM10 (Fig. 1C).



(A) Total DNA isolated from *Bacillus thuringiensis* isolates

(B) *cry*1 profile

(C) *cry*4 profile

FIGURE 1. Cry gene profile of native *Bacillus thuringiensis* isolates.

DISCUSSION

The insecticidal activity of *B. thuringiensis* crystal protein was traditionally investigated by crude preparation of spore–crystal mixtures. In this study, crystal proteins of native bacterial isolates were compared with that of the reference strain HD1. The mortality response of larvae of *D. indica* showed high variability among the isolates.

TABLE 2. Details of amplicons obtained in *cry1* and *cry4* gene profile

Isolate	Details of amplicons					
	*cry*1			*cry*4		
	No. of bands observed	Mol. wt of amplicon (bp)	Expected size of amplicon (bp)	No. of bands observed	Mol.wt of amplicon (bp)	Expected size of amplicon (bp)
KY1	–	–	–	1	575	439
KY2	1	~250	277	–	–	–
KY3	1	~250	277	–	–	–
KY4	–	–	–	–	–	–
KY5	1	~250	277	–	–	–
KY6	1	~250	277	–	–	–
KK7	1	~250	277	–	–	–
KK8	1	~250	277	–	–	–
KK9	1	~250	277	–	–	–
EM10	–	–	–	3	531, 942, 1245	439
EM11	1	~250	277	–	–	–
HD1	1	~300	277	–	–	–
4Q1	–	–	–	1	524	439

Native isolates showed a lower mortality rate on test larvae as compared to reference strain HD1. All native isolates except EM11 recorded nearly 90% mortality in eight days. These could be considered as efficient isolates, according to Valicente and Barreto (2003) who reported that strains that killed more than 70% of larvae on 8th day were efficient ones. Some workers (Benhard *et al.*, 1997; Hossain *et al.*, 1997) have ranked bacterial isolates that caused more than 50% mortality when compared to standard strains as highly efficient and toxic (Chak *et al.*, 1994).

Author: Benhard et al. *'1994' or '1997'*

Statistical analysis of data carried out using Kendall's coefficient of concordance provides a measure of agreement or concordance between sample ranking or dependence of the samples (Siegal, 1975). Isolates ranked based on the mortality on 5th and 8th days confirmed that reference strain HD1 was more efficient in controlling larvae of *D. indica*. Among the native isolates, KK7, KK8, KK9 and KY2 were more efficient than the rest. Manimegalai *et al.* (2004) reported that susceptibility of lepidopteran larvae to parasporal crystal from various isolates of *B. thuringiensis* is one aspect of high selectivity of delta endotoxins towards insect species. Another reason for varying toxicity may be that certain insects have a low susceptibility for bacterial crystal proteins owing to the inefficient solubilization of crystals in the midgut. The delta-endotoxin is released by the bacterial isolate as protoxin and it should be converted to the toxic form, by the digestive enzymes in the midgut of insect larvae. Solubilization of crystal significantly enhances the toxic activity (Jaquet *et al.*, 1987). Since *B. thuringiensis* strains simultaneously produce more than one type of crystal proteins, bioassays can

be greatly influenced by the relative proportion of different proteins within the crystal. Hence it is difficult to accurately determine the spectrum of individual proteins causing toxicity.

Analysis of the potential of the strains against different orders of insect by bioassay is an exhaustive but time consuming process (Rajesh *et al.*, 2006). Of the alternative methods available, PCR analysis is considered to be good choice, as it allows rapid determination of the specific *cry* genes and has high sensitivity (Ceron *et al.*, 1995). The PCR amplification carried out with 11 isolates using *cry*1 gene specific primer indicated the presence of *cry*1 gene in the isolates KY2, KY3, KY5, KY6, KK7 KK8, KK9 and EM11 (Table 2). The *cry*1 gene is reported to be toxic to lepidopteran larvae. No amplification was obtained for isolates KY1, KY4 and EM10 indicating that they may contain cry gene other than *cry*1 gene group since they were also found toxic to *D. indica*. Isolates KY1 and EM10 and the reference strain 4Q1 showed amplification of cry4 gene alone indicating their potential against dipteran insects and presence of other gene not identified in the present investigation. Salem *et al.* (2006) identified *cry*1 and *cry*4 gene by producing fragments of 277 bp for *cry*1 and 439 bp for *cry*4 genes. Similar observations had been made by earlier workers (Carrozi *et al.*, 1991; Chak *et al.*, 1994). However, isolate KY4 remained negative for the amplification of *cry*1 and *cry*4 genes, and the mortality caused by the isolate can be attributed to the presence of gene other than *cry*1 and *cry*4 gene group which has to be investigated in future work. The genetic diversity and distribution of *cry* genes varied according to the regions from where they were collected. Thus the PCR analysis was not in full agreement with the results of the bioassay studies. Carrozi *et al.* (1991) suggested that novel isolates containing novel *cry* genes may give PCR products different in size relative to the standard or may completely lack PCR products. The report of 32 different *cry* gene groups by Crickmore *et al.* (1998) suggested that for identification of specific *cry* gene groups, the isolates should be examined using different sets of *cry* gene primers and also with primers specific for subgroups of each *cry* gene.

To conclude, the most obvious factors that contribute to the potency of delta-endotoxins are the origins of toxin (strain), the ability of gut juice to dissolve the protoxins and the intrinsic susceptibility of the insect to the toxin. The present study resulted in the identification of insecticidal activity of native isolates of *B. thuringiensis* active against *D. indica* and the presence of *cry*1 gene in eight of the 11 isolates studied. Two isolates had only *cry*4 gene and were still toxic to *D. indica* and one with neither *cry*1 or *cry*4 gene also killed the larvae. Emphasis must hence be laid on the identification and isolation of novel *cry* genes from bacterial strains which will be useful in pyramiding of different resistant genes.

ACKNOWLEDGEMENTS

The financial assistance from the Department of Biotechnology, Government of India and facilities provided by Kerala Agricultural University are thankfully acknowledged.

REFERENCES

Ben Dov E. A., Zaritsky E., Dahan Z., Barak R., Sinai R., Manasherob A., Khamraev E., Troitskaya A., Dubitsky N. B. and Margalith (1997) Extended screening by PCR for seven *cry* group genes from field collected strains of *Bacillus thuringiensis*. Applied and Environmental Microbiology, 63: 4883–4890.

Benhard K., Jarret P., Meadows M., Butt J., Ellis D. J., Roberts G. M., Pauli S., Rodgers P. and Burges H. D. (1997) Natural isolates of *Bacillus thuringiensis*: Worldwide distribution, characterization and activity against insect pests. Journal of Invertebrate Pathology, 70: 59–68.

Carrozi N., Kramer V., Warren G., Evola S. and Koziel M. (1991) Prediction of insecticidal activity of *Bacillus thuringiensis* strains by polymerase chain reaction product profiles. Applied and Environmental Microbiology, 57(11): 3057–3061.

Ceron J., Ortiz A., Quintero R., Guereca L. and Bravo A. (1995) Specific PCR primers directed to identify *CryII* and *CryIII* genes within a *Bacillus thuringiensis* strain collection. American Society of Microbiology, 61: 3826–3832.

Chak K. F., Chao D. C., Tseng M. Y., Kao S. S., Tuan S. J. and Feng T. Y. (1994) Determination of *cry* type genes of *Bacillus thuringiensis* isolates from Taiwan. Applied and Environmental Microbiology, 60: 2415–2420.

Crickmore N., Zeigler D. R., Feitelson J., Schnepf E., Van Rie J., Lereclus D., Baum J. and Dean D. H. (1998) Revison of nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. Microbiology and Molecular Biology Reviews, 62: 807–813.

Dulmage H. T. (1970) Insecticidal activity of HD1, a new isolate of *Bacillus thuringiensis var. alesti*. Journal of Invertebrate Pathology, 15: 232–239.

Fietelson J. S. (1993) The *Bacillus thuringiensis* family tree. In: *Advanced engineered pesticides*, Kim L. and Marcel (Eds). Dekker Inc., NY, USA, 63–71.

Hossain M. A., Ahmed S. and Hoque S. (1997) Abundance and distribution of *Bacillus thuringiensis* in the agricultural soils of Bangladesh. Journal of Invertebrate Pathology, 70: 221–225.

Iriarte J., Dumanoir V. C., Bel Y., Porcar M., Ferrandis M. D., Lecadet M-M., Ferré J. and Caballero P. (2000) Characterization of Bacillus thuringiensis ser. Balearica (Serotype H48) and ser. Navarrensis (Serotype H50): two novel serovars in Spain. Current Microbiology, 40: 17–22.

Jaquet F., Hutter R. and Luthy P. (1987) Specificity of *Bacillus thuringiensis* delta endotoxin. Applied and Environmental Microbiology, 53: 500–504.

Kendall M. G. and Smith B. (1939) The problem of m Rankings. The Annual of Mathematical Statistics, 10(3): 275–287.

Kronstad J. W. and Whiteley H. R. (1986) Three classes of homologous *Bacillus thuringiensis* crystal-protein genes. Gene, 43: 29–40.

Lambert B., Hofte H., Annys K., Jansens S., Soetaert P. and Peferoen M. (1996) Novel *Bacillus thuringiensis* insecticidal protein with a silent activity against Coleopteran larvae. Bioscience, 42: 112–122.

Manimegalai S., Chandramohan N. and Jayarani S. (2004) Sex influenced Inheritance of *Bacillus thuringiensis cry*1Ac Resistance in American Bollworm *Helicoverpa armigera* (Hubner). Pesticide Research Journal, 16(4): 40–44.

Rajesh N. R., Alagawadi A. R. and Krishnaraj P. U. (2006) *Cry* diversity of *Bacillus thuringiensis* isolates of Western Ghat region and their bioefficacy against *Spodoptera litura* and *Helicoverpa armigera*. Entomon, 31(2): 89–98.

Salem H. H., Ali B. A., Tian H. H. and Qing D. X. (2006) Molecular characterization of novel *Bacillus thuringiensis* isolate with molluscicidal activity against the intermediate host of Schistosomes. Biotechnology, 5(4): 413–420.

Sambrook J. and Russel D. W. (2001) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York, USA, 1541 pp.

Schesser J. H., Kramer K. J. and Bulla L. A. (1977) Bioassay for homogeneous parasporal crystal of *Bacillus thuringiensis* using the tobacco hornworm, Manduca sexta. Applied and Environmental Microbiology, 33: 878–880.

Siegal S. (1975) *Non Parametric Statistical Inference*, Marcel, Dekker, New York, 217 pp.

Travers S. R., Martin P. A. M. and Reichelderfer C. F. (1987) Selective process for efficient isolation of soil *Bacillus* spp. Applied and Environmental Microbiology, 53(6): 1263–1266.

Valicente F. H. and Barreto M. R. (2003) *Bacillus thuringiensis* survey in Brazil: Geographical distribution and insecticidal activity against *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera:Noctuidae). Neotropical Entomolgy, 32(4): 639–644.

(Received 6 November 2009; accepted 15 November 2010)