International Journal of Engineering and TechnologyVol. 1, No.4 ,October, 2009
ISSN: 1793-8236

Distribution of Bacillus thuringiensis in Gunung Jerai forest (Malaysia)with insecticidal activity against lepidopteran and dipteran insects

Reena Josephine, C.M1, Sreeramanan2 and Lee Yean Wang3 and Xavier, R1

Abstract—**Globally there has been a concerted effort to isolate Bt strains from different environments for use as microbial pesticides and in transgenic plants. In our study, 106 soil samples were collected from different altitude in Gunung Jerai forest, an undisturbed rain forest ecosystem, Kedah Darul Aman State, Malaysia. Preliminary screening included temperature selection methods to isolate Bacillus–like colonies, followed by phase contrast microscopy to screen presumptive Bt isolates. Twenty three isolates were selected for PCR analysis with Cry1 general primer (gcry1), PCR to identify the presence of cry1-type genes. Out of 23 isolates, only 17 isolates showed the presence of cry1-type genes with the product size of 558 bp. Subsequently, the 17 Bt isolates which showed the presence of cry1-type genes were subjected to pathotype PCR to predict the insecticidal activity against lepidopteran pests. The results of the pathotype PCR revealed that out of 17 Bt isolates, only 11 isolates showed PCR product indicating their possible anti lepidopteran activity. Plasmid profiling studies indicated the presence of a major plasmid of size 22.5 to 28.5kb.Then the hemolytic positive strains were subjected to one step bioassay against the second instar larvae of Aedes aegypti. One Bt isolate, Bt 02, showed elevated larvicidal activity, which was compared to that of the reference strain Bt.subsp. israelensis. The crystal protein studies of the Bt isolate 02 indicated the presence of cuboidal crystals. SDS-PAGE analysis of the Bt isolate 02 showed a major protein of 101.77 kDa. However, presence of a protein of 32.68 kDa, indicted the possible presence of cytolytic toxin. Finally to study the diversity, two Bt isolates, Bt 22 and Bt 42 were subjected to partial DNA sequencing of the PCR amplicon. The results indicated that these two strains have different nucleotide sequences and each strain showed homology with the reported Bt strains which are also different from each other, thus indicating the presence diverse Bt strains in this environment. The Bt isolates showing anti lepidopteran and anti dipteran activity can be used in integrated pest management to control agricultural pests and to control the selected mosquito vectors.**

I. INTRODUCTION

Among all other problems, insect pests are continued to be the major limiting factor in agricultural production. It has been estimated that up to 15% of crops world wide are lost because of insect damage alone (Boulter et al., 1989). Indiscriminate and uncontrolled application of synthetic pesticides for the control of agriculturally important insect pests, although efficacious in most cases, has severe drawbacks and led to the development of insect resistance, resurgence, the emergence of secondary pests, development of new biotypes, the impact on nontarget organisms, environmental pollution, and residues in the agricultural products and animals (Nester et al., 2002). Among all other microbial pesticides, the most important biopesticides are those which utilize strains of the bacterium Bacillus thuringiensis. Consequently, the bacteria can be harnessed as insect pathogen for the use not only in agriculture but also in silviculture and public health control (Whitten and Oakeshott, 1990). In recent times interest in the use of B. thuringiensis products as an alternative to chemical insecticides has contributed so much that many research centers focused its efforts in the isolation of native strains of B. thuringiensis, leading to the establishment of B. thuringiensis strain collection worldwide. The establishment of these collections has contributed to the present knowledge of the ecological distribution of these microorganisms in nature. The objective of the current investigation focuses on the isolation and characterization of native B.thuringiensis isolates from Gunung Jerai forest, a rain forest ecosystem in Malaysia.

Reena Josephine, C.M is with the AMIST University Semeling, 08100 Bedong, Kedah Darul Aman, Malaysia

Sreeramanan is with School of Biological Sciences, Universiti Sains Malaysia.

Lee Yean Wang is with the Coopers Lifesci Sdn.Bhd.-WellTech Healthcare Group, Kuala Lumpur, Malaysia

Xavier, R is with the AMIST University Semeling, 08100 Bedong, Kedah Darul Aman, Malaysia.

II. MATERIALS AND METHODS

A. Bacterial strains

B. thuringiensis subsp. israelensis was obtained from Institute for Medical Research, Kuala Lumpur, Malaysia. B. thuringiensis subsp. kurstaki (Dipel) was kindly provided by Prof. Dr. V. Murugan, Centre for Biotechnology, Anna University, Chennai, India.

B. Sample collection

One hundred and six soil samples were collected at different altitudes of Gunung Jerai forest, Kedah Darul Aman, Malaysia. To our knowledge, this forest had not been previously treated with B. thuringiensis based biopesticides, thus it is unlikely that commercial strains of B. thuringiensis were an artifact in this study. Samples were collected by scrapping off the surface material with a sterile spatula and collecting approximately 50 g sample 2-5 cm below the

surface in sterile plastic bags, which were then stored at 4 °C until further processing.

A. *Isolation of Bacillus thuringiensis*

The heat shocked aliquots were serially diluted to10-9, plated on nutrient agar and incubated overnight at 30 °C. Based on Bacillus colonial morphology, colonies were selected at random and sub cultured in nutrient agar medium for sporulation. Initially, the cultures were observed under light microscope for the rod shaped vegetative cells, motility and sporulation.

B. *Coomassie Brilliant Blue (CBB) staining*

A straight inoculating wire was used to transfer an aliquot of a sporulated colony on to a microscope slide. The slide was then heat fixed and stained (0.133 % Coomassie Brilliant Blue stain in 50 % acetic acid), rinsed with distilled water, dried and observed under light microscope using a 100x oil immersion objective (Rampersad and Ammons, 2002). The presence of parasporal bodies were clearly observed as ene Laboratories Sdn, BhD, Kuala Lumpur, Malaysia.

dark-blue staining objects.

C. *Crystal Morphology*

The environmental isolates of B. thuringiensis were grown on nutrient broth for 72 h or until sporulation and auto lysis. The parasporal inclusions were examined after cell lysis by CBB staining and viewed under light microscope (Olympus BX50 fitted with a JVC K-F55B color video camera and analysis Docu Version 3.1 image analysis system). Crystal morphology was classified as one of the four types, bipyramidal (B), spherical (S), cuboidal (C) and irregular –shaped (I).

D. *Oligonucleotide primers used*

Oligonucleotide primers were as described by Bravo *et al.* (1998) and Carozzi *et al*. (1991) and synthesized by Medig

Table 1: *cry*1 specific primers used in PCR screening

Primer pair	Positions	Product Size (Kb)	Sequence	Reference
gral-cry1	1472-2029	0.558	5’CTGGATTTACAGGTGGGGATAT3’(d) 5’TGAGTCGCTTCGCATATTTGACT3’(r)	Bravo *et al.* (1998)
Lep 1A Lep 2B	310-330 3046-3066	2.7	5’CCGGTGCTGGATTTGTGTTA3’ (d) 5’TACATGCCCTTTCACGTTCC3’ (r)	Carozzi *et al.* (1991)

A. *Bioassay: Toxicity of Bacillus thuringiensis isolates to Aedes aegypti*

Peliminary one-dose bioassay was conducted with four-day old larvae of the mosquito Aedes aegypti (Diptera: Culicidae), the vector of dengue virus, at a fixed dose of 250 µg/ml of spore- crystal mixture and B. thuringiensis subsp. israelensis was used as positive control. Hemolytic positive B. thuringiensis isolates were grown in 100 ml nutrient broth medium until 95 % of cell lysis. The sporulated cultures were harvested by centrifugation at 6000 rpm for 15 min. The spore-crystal mixture was washed twice with sterile distilled water to remove the β- exotoxin. Ten 4-day-old larvae were introduced into 5 ml of the cell suspension. Similarly, the larvae were also introduced in distilled water without spore crystal mixture that served as negative control. The tests were performed in triplicate. The larval mortality was scored after incubation at 25 °C for every 12 h.

III. RESULTS AND DISCUSSION

A. *Colonial Morphology*

The colonial morphology of Bacillus thuringiensis and Bacillus cereus cannot be distinguished. Therefore colonies showing the typical morphology of B. cereus and B. thuringiensis (De Respinis et al., 2006) were selected. The fully developed colonies are round, white, with regular margins exhibiting identical colonial morphology as that of the wild type B. thuringiensis strains. The organism in the Fig.

1 and Fig. 2 was identified to be B. thuringiensis isolate 42 based on the presence of crystal proteins.

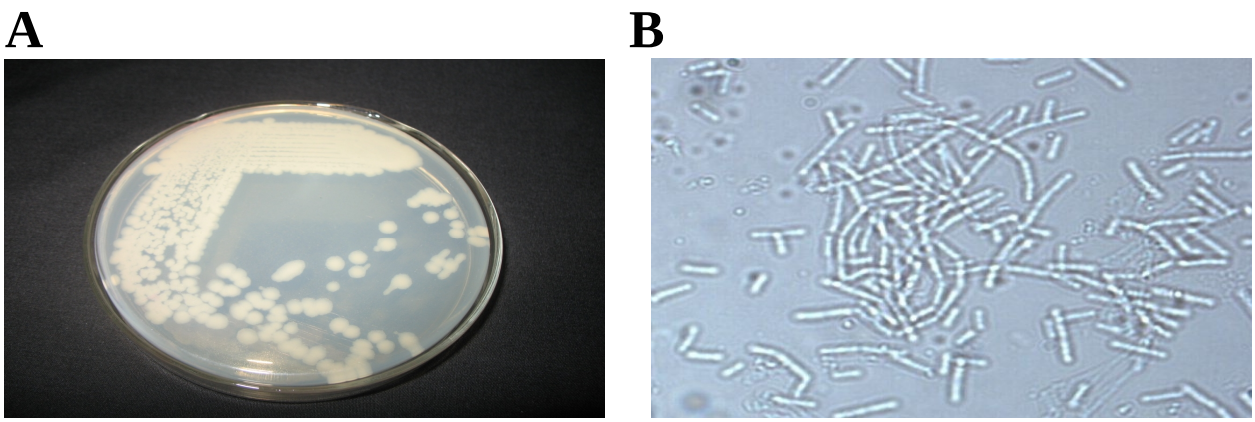


Fig. 1: Colony morphology, (A) Colonies of Bacillus thuringiensis on nutrient agar; (B) Vegetative cell morphology of Bacillus thuringiensis

A. Coomassie Brilliant Blue (CBB) staining and Crystal protein morphology

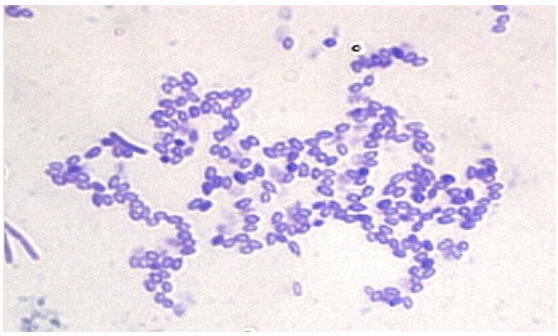
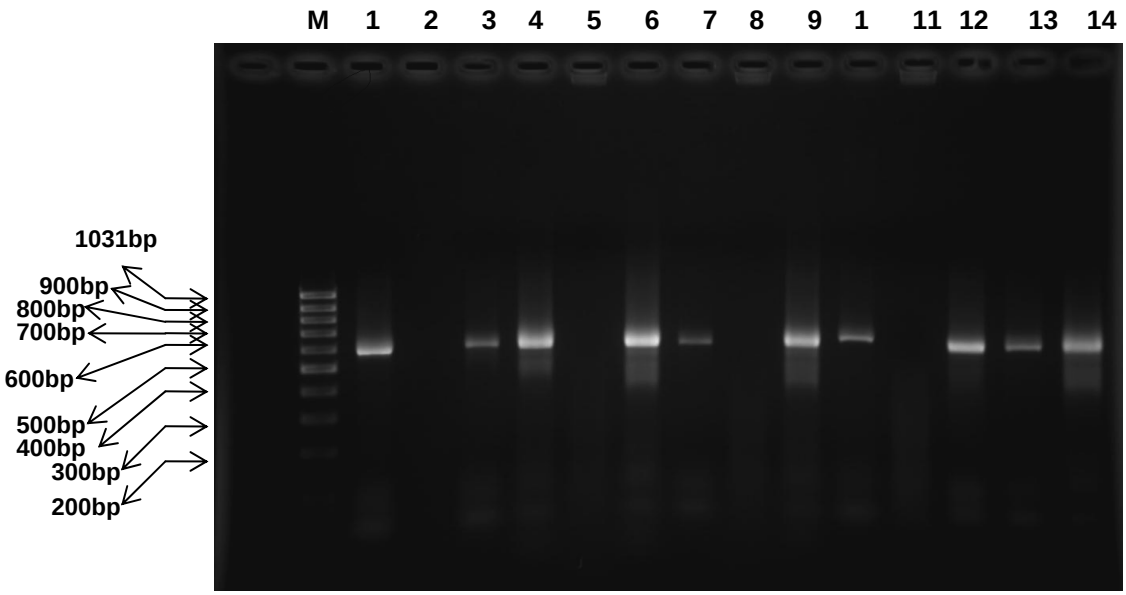


Fig. 2: Spores and crystals of Bacillus thuringiensis isolate 42

A. PCR amplification using cry1 general primers

(A)



(B)

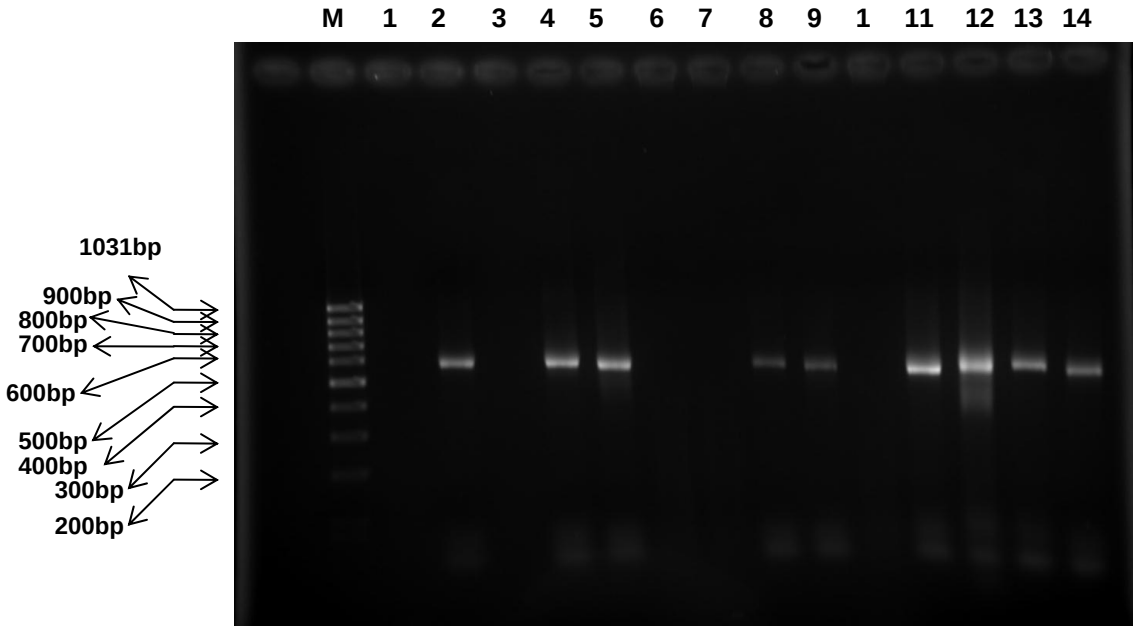
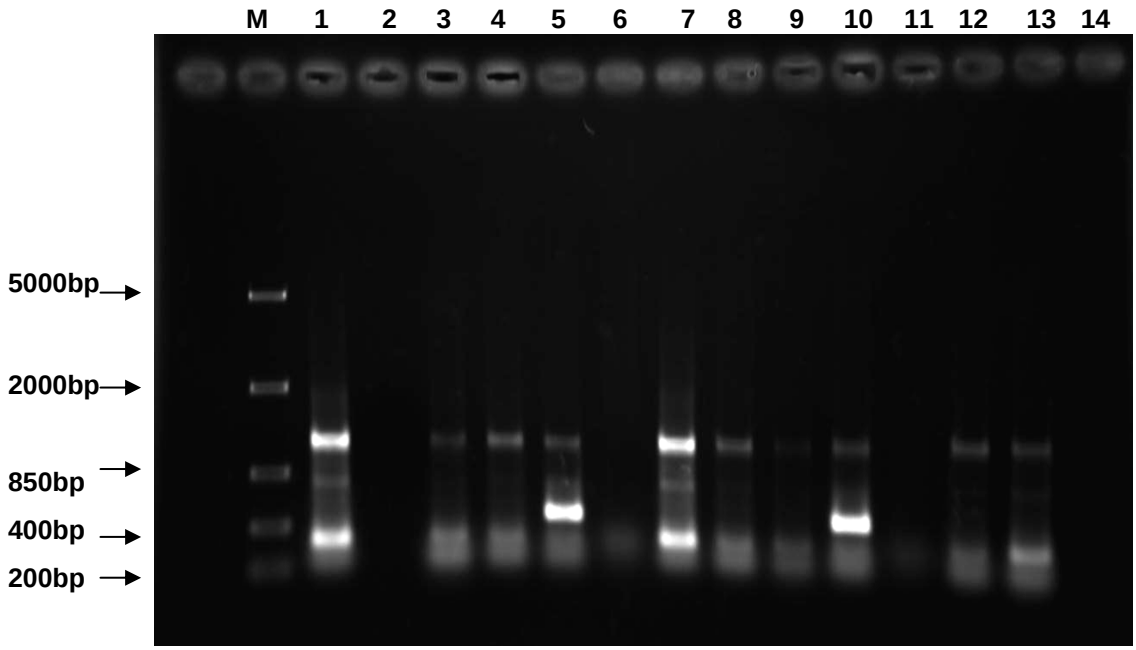


Fig. 3: Agarose gel electrophoresis analysis of PCR products using cry1 general primers. (A) M-Marker DNA (Mass ruler DNA ladder low range SM#0383) Lane 1- Bacillus thuringiensis subsp. kurstaki (Dipel) Lane 2- PCR mix without template DNA Lanes 3 -14 - Amplified and unamplified isolates of Bacillus thuringiensis; (b) M-Marker DNA (Mass ruler DNA ladder low range SM#0383) Lane 1- PCR mix without template DNA Lane 2-Bacillus thuringiensis subsp. kurstaki (Dipel) Lanes 4-14: Amplified and unamplified isolates of Bacillus thuringiensis.

A. PCR amplification using cry1 pathotype primers



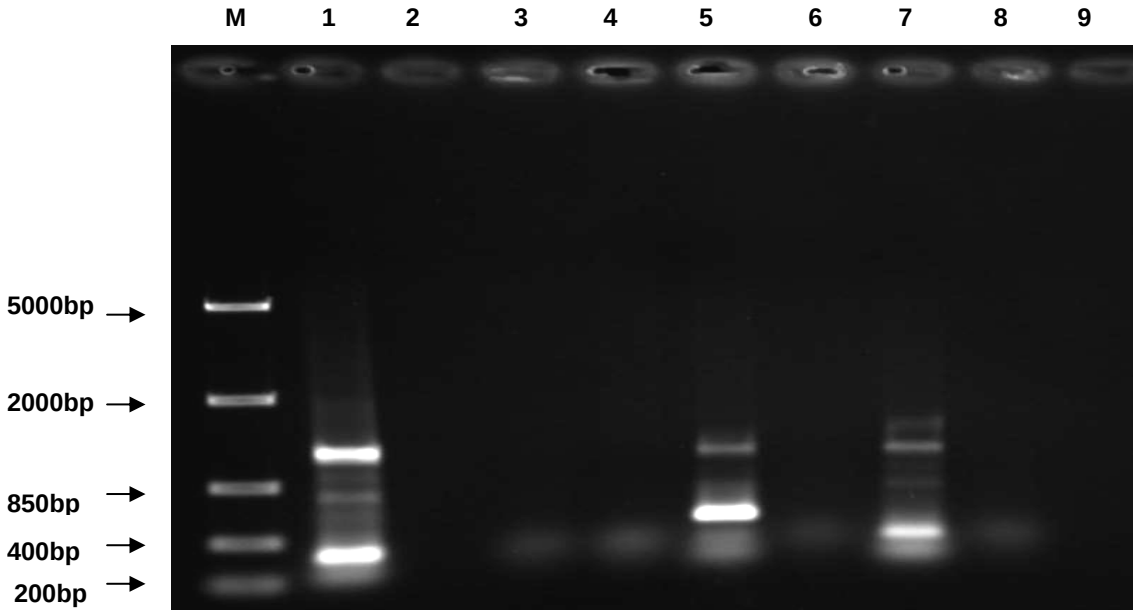


Fig. 4. Agarose gel electrophoresis analysis of PCR products using *cry*1 pathotype primers (a). M-Fast ruler DNA ladder middle range SM#11 Lane 1- *Bacillus thuringiensis* subsp. *kurstaki* (Dipel) Lane 2- PCR mix without template DNA Lanes 3 -13 - Amplified and unamplified isolates of *Bacillus thuringiensis* ; (b). M-Fast ruler DNA ladder middle range SM#1113 Lane 1- *Bacillus thuringiensis* subsp. *kurstaki* (Dipel)Lane 2- PCR mix without template DNA Lanes 3-8, Amplified and unamplified isolates of *Bacillus thuringiensis.*

All the 23 B. thuringiensis isolates were examined by PCR for the presence of cry1-type genes. Bravo et al. (1998) designed a general primer (gcry1) to amplify specific fragments of cry1-type genes. The predicted size of the PCR-amplified product is 558bp. In this analysis rapid cell lysates were used as DNA template. This method enables analysis of several samples at the same time and reduced the possibility of sample contamination. However, there could be intrinsic variation in the quantity of the PCR product because of the lack of standardized amount of DNA in the PCR mixture (Ceron et al., 1995). The presence of a PCR product of the expected size indicated that the corresponding gene is present in the B. thuringiensis isolates. Out of 23 isolates only 17 isolates showed the presence of cry1-type genes with the product size of approximately 558 bp (Fig. 3 a, b) , which is in agreement with earlier reports.

It is important to search for novel insecticidal proteins that will help control the insect pests. The 17 B. thuringiensis isolates which amplified for cry1 general primers were used for further molecular characterization by pathotype PCR. The rapid cell lysates from the cry1 general PCR positive B. thuringiensis isolates were used as DNA template. Carozzi et al. (1991) designed a set of Lepidopteran specific primers, to predict the insecticidal activity of B. thuringiensis isolates. From this pathotype specific oligonucleotide primers, a forward primer (Lep 1A) was selected from one set and a reverse primer (Lep 2B) was selected from other set, in order to probe the most conserved regions of all known cry1 type gene sequences present in the B. thuringiensis isolates. Thus the amplified PCR product may contain all possible cry1 gene sequences. Theoretically the product size was predicted to be 2.7 kb (Xavier et al., 2007). The results of the pathotype-PCR revealed that out of 17 B. thuringiensis isolates, only 11 isolates showed PCR product and the size of the product is approximately 1.2 kb (Fig. 4 a, b). It is presumed that these B. thuringiensis isolates should exhibit entomocidal activity, however, with varying degrees. Depending upon the type of cry1 gene, the same primer may generate two different PCR amplicons (Carozzi, et al., 1991) The toxicity of a B. thuringiensis isolate is primarily depend on the cry gene content, and the toxicity shall vary among different types of Lepidopteran pests. Novel isolates not containing known genes might give PCR products different in size relative to the standard or might completely lack PCR products (Carozzi et al., 1991). Hence, a preliminary bioassay, followed by a dose-response bioassay with these pathotype positive isolates with agronomically important polyphagous pests would reveal the entomocidal nature of these isolates.

A. Mosquito bioassay

Mosquito-borne diseases such as malaria, filariasis, dengue and viral encephalitis contribute to a large proportion of health problems of developing countries. Vector control is the effective option to manage these diseases (Service, 1986). Hemolytic positive strains were subjected to preliminary one step bioassay (Ohba, 1996). The results in the preliminary bioassay showed that out of 23 B. thuringiensis isolates, only one isolate, isolate 2, showed elevated larvicidal activity against Aedes aegypti, which was comparable to that of the reference strain B. thuringiensis subsp. israelensis.

IV. CONCLUSION

To conclude, the study enabled the isolation of one B. thuringiensis isolate 2 possessing antidipteran activity. Among the pathotype positive isolates, two isolates namely

isolate 22 and isolate 42, were studied in detail. These antidipteran and antilepidopteran B. thuringiensis isolates as determined by bioassay and predicted through pathotype PCR, respectively can play a major role in agriculture (Integrated Pest Management) and health care (Integrated Vector Management).

REFERENCES

[1] Boulter, D., Gatehouse A.M.R. and Hilder, V. (1989). Use of cowpea trypsin inhibitor (CPTI) to protect plants against insect predation. Biotechnol. Adv, 7:489-497.

[2] Nester, E.W., Thomashow, S., Metz, M. and Gordon, M. (2002). 100 years of Bacillus thuringiensis: a critical scientific assessment [on]. American Society for Microbiology, Washington, D.C. http:/www.asmusa.org.line.

[3] Whitten, M.J. and Oakeshott, J.G. (1990). Biocontrol of Insects and weeds. In: Persley, G.J. editor, (1990). Agricultural Biotehnology, CAB International, Wallingford, pp. 123-142

[4] Rampersad, J. and Ammons, D. (2002). Usefulness of staining parasporal bodies when screening for Bacillus thuringiensis. J Inverteb Pathol, 79:203-204.

[5] Carozzi, N.B., Kramer, V.C., Warren, G.W., Evola, S. and Koziel, M.G. (1991). Prediction of insecticidal activity of Bacillus thuringiensis strains by polymerase chain reaction product profiles. Appl. Environ. Microbiol, 57:3057-3061.

[6] Bravo, A., Sarabia, S., Lopez, L., Ontiveros, H., Abarca, C., Ortiz, A., Ortiz, M., Lina, L., Villalobos, Pena, G., Nunez Valdez, M.E., Soberon, M. and Quintero, R. (1998). Characterization of cry genes in a Mexican Bacillus thuringiensis strain collection. Appl Environ Microbiol, 64:4965-4972.

[7] De Respinis, S., Demarta, A., Patocchi, N., Luthy, P., Peduzzi, R. and Tonolla, M. (2006). Molecular identification of Bacillus thuringiensis var. israelensis to trace its fate after application as a biological insecticide in wetland ecosystem. Lett. App. Microbiol, 43:495-501.

[8] Ceron, J., Ortiz, A., Quintero, R., Guereca, L. and Bravo, A. (1995). Specific PCR primers directed to identify cry I and cry III genes within a Bacillus thuringiensis strain collection. Appl. Environ. Microbiol, 61: 3826-3831.

[9] Xavier, R., Nagarathinam, P., Gopalakrishnan, Murugan, V. and Jayaraman, K. (2007). Isolation of Lepidopteran Active Native Bacillus thuringiensis strains Through PCR Panning. Asia Pacific Journal of Molecular Biology and Biotechnology, 15(2): 61-67.

[10] Service, M.W. (ed). (1986). Blood-sucking insects: Vectors of disease. Edward Arnold, London, United Kingdom.

[11] Ohba, M. (1996). Bacillus thuringiensis populations naturally occurring on mulberry leaves: a possible source of the populations associated with silkworm-rearing insectaries. J. Appl. Bacteriol, 80: 56-64.