Bio-efficacy and molecular characterization of the native isolates of *Bacillus thuringiensis* Berliner

By

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THESIS

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DECLARATION

I, hereby declare that this thesis entitled **"Bio-efficacy and molecular characterization of the native isolates of *Bacillus thuringiensis* Berliner"** is a *bonafide* record of research work done by me during the course of research and that it has not been previously formed the basis for the award to me of any degree, diploma, fellowship or other similar title, of any other University or Society.

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CERTIFICATE

Certified that this thesis entitled **"Bio-efficacy and molecular characterization of the native isolates of *Bacillus thuringiensis* Berliner"** is a *bonafide* record of research work done independently by **Miss. Jyothi Sara Jacob** under my guidance and supervision and that it has not formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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Jyothi Sara Jacob

Dedicated to

Appachen & Ammachi

Appa & Amma

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Abbreviations

cm	Centimeter
CPBMB	Centre for Plant Biotechnology and Molecular Biology
DNA	Deoxyribonucleic acid
dNTP	Deoxy Nucleotide Triphosphate
EDTA	Ethylene Diamine Tetra Acetic Acid
g	Gram
ICP	Insecticidal Crystal Protein
kDa	kilo Dalton
LB	Luria Bertani
LC_{50}	Median Lethal Concentration
LT_{50}	Median Lethal Time
mDa	mega Dalton
ml	Millilitre
mM	Millimole
µg	Microgram
µl	Microlitre
ng	Nanogram
OD	Optical Density
PCR	Polymerase Chain Reaction
pH	Hydrogen ion concentration
%	Per cent
RAPD	Random amplified Polymorphic DNA
RNase	Ribonuclease
rpm	Rotations per minute
SDS	Sodium dodecyl sulphate
TAE	Tris Acetate EDTA
U	Unit
UV	Ultra Violet
V	Volts
Vip	Vegetative Insecticidal Protein
v/v	Volume by volume
w/v	weight by volume

Introduction

1. INTRODUCTION

In the past half century, control of insect pests has relied almost exclusively on the use of synthetic organic insecticides. The application of insecticides not only contributed to the effective management of insect pests but also led to the development of insect resistance against insecticides, resurgence of sucking pests, contamination of soil, ground water and food materials. On considering the severe ill effects associated with the promiscuous use of insecticides in agriculture, there is an urgent need to minimize the use of synthetic chemical insecticides for the management of insect pests. For the increasing public concern over health hazards of synthetic organic pesticides and the incredible spiralling increase on the cost of cultivation, biopesticides seem to be one of the best alternatives for pest management.

The use of biopesticides, utilizing insect pathogens, is gaining world wide importance in IPM. Biopesticides are the important components in ecofriendly pest management. The use of biological control methods for insect pest suppression has been accepted and proved safe world wide. The use of insect pathogens for the management of insect pests is gaining much importance.

Microbial agents are primary means of biological control of insect pests. The entomopathogenic bacterium, *Bacillus thuringiensis* Berliner is considered ideal for pest management because of its wide host range and specificity of different subspecies to different group of insects. The biocontrol activity of this bacterium is due to the insecticidal crystal proteins (ICP) or δ- endotoxins produced in the sporulating cells (Nagamatsu *et al*., 1998). The continuous use of *B. thuringiensis* based biopesticides increased the risk of resistance development. So isolation and screening of novel *B. thuringiensis* isolates are important.

Insecticides derived from *B. thuringiensis* constitute an attractive alternative to synthetic organic insecticides. *B. thuringiensis* based microbial pesticides comprise most of the microbial pesticide segment. *B. thuringiensis* toxins are extra ordinarily lethal to certain pests. The molecules of *B. thuringiensis* toxins are 80000 times more potent than pyrethroids (Feitelson *et al.*, 1992). Thousands of *B. thuringiensis* strains have been isolated with characteristic set of toxins. Screening procedures are being carried out for native isolates for increased virulence, tolerance to photo-inactivation, greater persistence on the phylloplane and also for the novel activity against additional group of insects.

A current concern regarding the use of *B. thuringiensis* is the development of resistance to its toxins in target pests. High levels of resistance to the δ- endotoxin of *B. thuringiensis* subsp. *kurstaki* have been recorded for the Indian meal moth, *Plodia interpunctella* Hubner (Mc Gaughey, 1985). The insect lines that developed resistance to a particular kind of crystal proteins of *B. thuringiensis* were found to be sensitive to a different kind of crystal protein of *B. thuringiensis*. Works are being continued throughout the world to isolate novel crystal proteins that are effective in killing *B. thuringiensis* resistant insect lines. Moreover the commercial preparations of *B. thuringiensis* from indigenous strains were reported to be more potent than imported ones and the indigenous native strains are considered to be more effective than non-indigenous ones. So the search for novel native isolates of *B. thuringiensis* is important.

The tobacco caterpillar, *Spodoptera litura* Fabricius (Lepidoptera: Noctuidae) is a polyphagous pest of crops like tobacco, cotton, vegetables and banana. This pest is distributed through out South and Eastern world tropics infesting 112 species of plants belonging to 44 families of which 40 species were known from India (Chari and Patel, 1983). The larvae can be mass reared in semi-synthetic diet also (Ballal, 2004).

Strains of *B. thuringiensis* isolated so far were found to be comparatively less pathogenic to *S. litura*. Hence, the present study aims to identify effective strains of *B. thuringiensis* from the native isolates and to study their genetic diversity. The objectives of the present investigation are the following.

1. Preliminary screening for pathogenicity of 20 native isolates of *B. thuringiensis* against *S. litura*.
2 Bioassay of the effective isolates of *B. thuringiensis* on the larvae of *S. litura*.
3 Molecular characterization of the efficient isolates of *B. thuringiensis* by RAPD- PCR technique.

Review of literature

2. REVIEW OF LITERATURE

Bacillus thuringiensis Berliner is an aerobic spore forming variant of *Bacillus cereus* that was described in the early 1900's (Berliner, 1915). It is a soil bacterium, which produces Insecticidal Crystal Proteins during sporulation. Usually one crystal per cell is present. Crystal shapes vary in different subspecies of *B. thuringiensis*. It includes bipyramidal, spherical, rhomboidal, triangular, irregular or flat (Hofte and Whitely, 1989). The crystalline inclusions consist of proteins, exhibiting a highly specific insecticidal activity (Aronson *et al*., 1986; Whitely and Schnepf, 1986).

2.1 History of *B. thuringiensis*

The first record of *B. thuringiensis* was given in 1901, when Ishiwata discovered a bacterium from diseased silkworm, *Bombyx mori* (Linnaeus) (Lepidoptera: Bombycidae) larva in Japan and was named as *Bacillus sotto* (Ishiwata, 1901). Between 1909 and 1912, Berliner, working at a research station for grain processing investigated an infectious disease of the Mediterranean flour moth (*Ephestia kuhniella* Zeller) (Lepidoptera: Pyralidae). The infected insects were originally obtained from a mill, in the district of Thuringia, Germany. He reported a spore forming bacterium as the causative agent and designated it as *Bacillus thuringiensis* Berliner (Berliner, 1915).

2.2 Classification of *B. thuringiensis*

The classification scheme to identify the crystalliferous bacteria based on their morphological and biochemical characteristics was proposed by Heimpel and Angus (1958). In the early 1960s, the classification of the *B. thuringiensis* subspecies based on the serological analysis of the flagella (H) antigens was introduced (de Barjac and Bonnefoi, 1962). Until 1977, only 13 subspecies had been described and all the subspecies were toxic to lepidopteran larvae only. Burges *et al*. (1982)

numbered and registered the new H-serotypes at the International Entomopathogenic Bacillus Centre (IEBC) at Institut Pasteur, Paris, France.

The bacterium, *B. thuringiensis* has been classified on the basis of cellular, cultural, biochemical and genetic characteristics (Hansen *et al*., 1998). By 1999, the wide diversity of *B. thuringiensis* strains was classified into 69 H serotypes and 82 serological varieties (Lecadet *et al*., 1999).

2.3 Distribution of *B. thuringiensis*

Most *B. thuringiensis* strains were isolated in association with insects (Ishiwata, 1901; Berliner, 1915; Dulmage, 1970; Dulmage and Aizawa, 1982; Krieg *et al*., 1983). Soils were the main source for the isolation of *B. thuringiensis* strains. The study conducted by de Lucca *et al*. (1981) showed that *B. thuringiensis* could be found in soil samples. Dulmage and Aizawa (1982) suggested that the normal habitat of *B. thuringiensis* was soil.

The bacterium is widely distributed in nature in other habitats also like insect cadavers (Kreig *et al*., 1983), stored products (de Lucca *et al*., 1982) and leaf surfaces (Smith and Couche, 1991).

Theunis *et al*. (1998) observed that the richest source of *B. thuringiensis* was grain dust from rice mills followed by rice field arthropods, soils and miscellaneous habitats. Hongyu *et al*. (2000) found an abundance *B. thuringiensis* abundance in ware house with average rate of isolation higher than soils.

2.4 Insecticidal activity of *B. thuringiensis*

The ability to produce crystalline inclusions during sporulation is the characteristic feature of *B. thuringiensis*. Most of the insecticidal activity of *B. thuringiensis* was associated with proteinaceous toxins located in parasporal inclusion bodies also known as parasporal crystals. These toxins account for upto 30 per cent of

the total protein content of the bacterium (Hofte and whitely, 1989; Aronson, 1993; Agaissi and Lereclus, 1995).

A number of insecticidal proteins expressed during the vegetative growth of *B. thuringiensis* had been identified (Estruch *et al*., 1996; Warren, 1997; Yu *et al*., 1997; Estruch and Yu, 2001; Selvapandiyan *et al*., 2001). These proteins called Vegetative Insecticidal Proteins (Vips) were reported from 15 per cent of the *B. thuringiensis* strains analysed (Estruch *et al*., 1996). The Vips have shown a broad insecticidal spectrum, including activity towards a wide variety of lepidopteran and coleopteran pests (Yu *et al*., 1997; Warren, 1997).

The spore of *B. thuringiensis* exhibited insecticidal activity (Somerville *et al*., 1970; Somerville and Pockett, 1975; Scherrer and Somerville, 1977) and the spore coat protein was appeared to be biochemically homologous to the parasporal crystal (Delafield *et al*., 1968; Somerville *et al*., 1968; Somerville, 1971; Lecadet and Dedonder, 1971; Lecadet *et al*., 1972).

Spores have been found to enhance the crystal activity in certain species of Lepidoptera (Li *et al*., 1987; Moar *et al*., 1989). According to Peferon (1997), *B. thuringiensis* spores could survive for several years where as the stability of crystal protein was highly variable lasting from a few days to several months depending upon the environmental conditions. Some synergistic effect was observed in many cases between the *B. thuringiensis* spores and toxins and the toxin free spores showed no activity (Li *et al*., 1987; Miyasono *et al*., 1994).

Johnson and McGaughey (1996) investigated the influence of *B. thuringiensis* subsp. *kurstaki* HD-1 spores upon the toxicity of purified Cry1Ab and Cry1C crystal proteins towards *B. thuringiensis* susceptible and resistant Indian Meal Moth (IMM), *P. interpunctella* larvae. With susceptible larvae, HD-1 spores were toxic in the absence of crystal protein and highly synergistic (approximately 35-50

fold) with either Cry1Ab or Cry1C protein with *B. thuringiensis* resistant IMM larvae. HD-1 spores were synergistic with Cry1Ab and Cry1C protein in all three resistant strains examined. Synergism was highest (approximately 25- 44 fold) in insects with primary resistance towards Cry1C (IMM larvae with resistance to subspecies *aizawai* or *entomocidus*). However HD-1 spores also synergized either Cry1Ab or Cry1C toxicity towards larvae resistant to *B. thuringiensis* subsp. *kurstaki* at a lower level (approximately 5-6 fold). The presence of spores reduced the time of death when combined with each of the purified Cry proteins, in case of susceptible larvae. Without spores the speed of intoxication and eventual death for larvae treated with Cry1C and Cry1Ab proteins was much slower than for the HD-1 preparation containing both spores and crystals together.

Liu *et al*. (1998) studied the synergism between *B. thuringiensis* spores and toxins against resistant and susceptible diamondback moth, *Plutella xylostella* (Linnaeus). They tested the effects of combination of *B. thuringiensis* spores and toxins on the mortality of larvae in leaf residue bioassays. Spores of *B. thuringiensis* subsp. *kurstaki* increased the toxicity of crystals to both resistant and susceptible larvae. For *B. thuringiensis* subsp. *kurstaki*, resistance ratios were 1,200 for a spore crystal mixture and 56,000 for crystals without spores. Synergism occurred between Cry2A and *B. thuringiensis* subsp. *kurstaki* spores against susceptible larvae and between Cry1C and *B. thuringiensis* subsp. *aizawai* spores against resistant and susceptible larvae. The result showed that *B. thuringiensis* toxins when combined with spores could be toxic even though the spores have little or no independent toxicity.

2.4.1 Signs and symptoms of *B. thuringiensis* infection

Most of the insecticidal activity of *B. thuringiensis* was associated with the proteinaceous toxins located in the parasporal bodies, also known as parasporal crystals. Heimpel and Angus (1956) have classified Lepidopteran larvae into three

types based on their susceptibility to crystalline endotoxin, bacterial spores or mixtures of the two.

Type I insects were killed by preparations of crystalline δ- endotoxin alone and the spores of the bacterium did not increase toxicity. Type II insects were susceptible to δ- endotoxin but the effect was enhanced by the presence of spores and Type III insects were killed only by the spore - endotoxin mixtures. The midgut pH of most susceptible larvae was found to be too alkaline to allow spore germination but suitable for dissolution and activation of protoxin. Thus most susceptible insects were characterized in Type I.

The first sign of poisoning following the ingestion of crystalline endotoxin by Type I insects was found to be paralysis of the gut and mouth parts leading to the cessation of feeding (Heimpel and Angus, 1956; Cooksey, 1971). These symptoms were appeared within minutes and its rapid onset, before dissolution and proteolitic activation of the protoxin suggested that it might be induced by intact protoxin. Subsequent to the onset of gut paralysis, there would be swelling of the microvilli present on the luminal surface of midgut epithelial cells, swelling of cells themselves, changes in the endoplasmic reticulum and mitochondria, disruption of ion and glucose transport and oxygen uptake, loss of adenosine triphosphate from midgut cells from the basement membrane and bursting of separated cells occured in the midgut lumen (Cooksey, 1971; Luthy, 1980; Burges, 1982; Dulmage and Aizawa, 1982).

Brunner and Stevens (1986) found that an infected caterpillar ceased to feed soon after ingestion of the bacterium and died after three to five days, dissolving into a soft, black biomass with a liquid exudate. Larvae became infected by ingesting spores from such cadavers or from the faeces of other infected larvae (Wilding, 1986).

2.4.2 Mode of action of *B. thuringiensis*

The mode of action of *B. thuringiensis* insecticidal proteins was thought to involve a number of steps leading to insect death within several hours following ingestion (Hofte and Whitely, 1989; Gill *et al*., 1992; Knowles, 1994; Powell *et al*., 1995).

The structure and mode of action of the lepidopteran active Cry1 proteins were extensively studied (Harvey *et al*., 1986; Ge *et al*., 1989; Bietlot *et al*., 1990; Choma and Kaplan, 1990; Knowles and Dow, 1993; Knowles, 1994).

The Cry1 proteins (protoxins) were found to be biologically inactive. The activity of the protoxin started only when it was ingested by the host insect and the solubilization of the protoxin was followed. A smaller 60-65 kDa activated protein was produced due to the cleavage by gut proteases and had recognized the specific binding site at the brush border membrane surface of the epithelial columnar cells lining gut lumen (Hofmann *et al*., 1988a, b; VanRie *et al*., 1989; Honee *et al*., 1991). This was followed by the pore formation, membrane transport disruption and cell lyses leading to insect death (Thomas and Ellar, 1983; Knowles and Ellar, 1987; Hofte and Whitely, 1989; Slatin *et al*., 1990; Schwartz *et al*., 1991, 1993).

Disruption of midgut structure and function lead to ion and pH imbalances in the haemolymph, total body paralysis and death of insect (Aronson *et al*., 1986).

2.5 Insecticidal Crystal Proteins (ICPs)

The most significant factor in determining the wide range of insect and even some non-insect species invaded by *B. thuringiensis* was the insecticidal proteins contained in parasporal crystals. Due to the production of insecticidal proteins, *B. thuringiensis* was considered as an opportunistic pathogen of insects (Jensen *et al*., 2003). The parasporal crystals formed in the cell constituted upto 35 per cent of the total dry weight. These crystalline inclusion bodies were the common

feature of the *B. thuringiensis* community and consisted of different ICPs or delta endotoxins each with its own unique specificity (VanFrankenhuysen and Nystrom, 2005). The delta endotoxins comprised of two multigenic families Cry (for Crystal) or Cyt (for Cytotoxic). Cry proteins were specifically toxic to different insect orders like Lepidoptera, Coleoptera, Hymenoptera, Diptera and to non-insect group, nematodes. In contrast, Cyt toxins were mostly found in *B. thuringiensis* strains active against dipteran insects (Bravo *et al*., 2005).

ICP genes were generally located on large plasmids, which could be exchanged between different *B. thuringiensis* strains during conjugation (Gonzalez *et al*., 1982). In addition, many of the ICP genes were part of transposon elements, which further added to their genetic nobility (Lereclus *et al*., 1984).

In addition, smaller binary insecticidal proteins related to the *B. thuringiensis sphaericus* dipteran specific Bin proteins had been isolated from different *B. thuringiensis* (Ellis *et al*., 2002). These were designated as Cry34/Cry35 and were tested to confer root protection to the western rootworm, *Diabrotica virgifera* LeConte (Coleoptera: Chrysomelidae) (Moellenbeck *et al*., 2001). Enzymes such as chitinases, proteases and phospholipase C further enhanced insect pathogenicity of *B. thuringiensis*.

2.5.1 Classification of ICPs

Hofte and Whitely (1989) divided the crystal proteins according to their activity spectrum. However, analysis and comparison of the nucleotide sequences showed that this approach did not necessarily reflect the evolutionary divergence between the different ICPs. In an effort to develop a uniform and standardized classification system for *B. thuringiensis* crystal proteins, Crickmore *et al*. (1998) based their nomenclature solely on amino acid sequence homology. Multiple bioassays were eliminated because of this and this system was recognized internationally. Currently 48 primary groups have been reported uniting proteins with

more than 45 per cent sequence similarity: Cry 1 to 46 and Cyt 1 and 2 (Crickmore *et al*., 2003).

2.5.2 Effect of Insecticidal Crystal Proteins on insects

Schesser *et al*. (1977) described a method for determining the toxicity of *B. thuringiensis* subsp. *kurstaki* parasporal crystal to the tobacco hornworm, *Manduca sexta* (Linnaeus). In the experiment larvae moulted to the second instar were tested. Two third of the larvae survived at a surface application of 5 ng per cm^2 and none survived at 62.8 ng per cm^2 after seven days. The mean lethal dose value for neonate *M. sexta* larvae was observed to be 6 ng per cm^2.

Schesser and Bulla (1979) determined the toxicity of *B. thuringiensis* parasporal crystal to the Indian Meal Moth (*P. interpunctella*). The numbers of insects killed were in relation to dry weight of crystals. Mortality was determined by comparing adult emergence in diets treated with crystals to emergence in untreated diets. There was only 30 per cent survival at an application rate of 0.414 µg per cm^2 and the LD_{50} value was found to be 0.299 µg per cm^2. The use of emergence data has provided a reliable and reproducible bioassay for comparing relative toxicities of crystals, spores and other cellular components to the insects.

Jaquet *et al*. (1987) determined the insecticidal activity of δ-endotoxins of 14 *B. thuringiensis* strains belonging to 12 subspecies against *Pieris brassicae* (Linnaeus), *Heliothis virescens* (Fabricius) and *Spodoptera litura* (Fabricius). Larvae of *P. brassicae* were highly susceptible to purified crystals of strains of *B. thuringiensis* subsp. *thuringiensis* and *B. thuringiensis* subsp. *morrisoni*. *H. virescens* responded best to *B. thuringiensis* subsp. *kenyae* and *B. thuringiensis* subsp. *kurstaki*. The crystals of *B. thuringiensis* subsp. *entomocidus* strain were found to be potent against *S. litura*. In the experiment, they found that the predissolution of the crystal enhanced the insecticidal activity against *H. virescens.*

Sheikh *et al*. (1990) conducted a bioassay on the first instar larvae of pink boll worm, *Pectinophora gossypiella* (Saunders) using δ-endotoxins or purified crystals of an indigenous isolate of *B. thuringiensis* subsp. *sotto*. The LD_{50} of the target insect was determined to be 30 µg per ml of the purified δ-endotoxins per milliliter of the diet.

Bing *et al*. (1995) conducted SDS-PAGE analysis of the δ-endotoxins crystals of *B. thuringiensis* HD-1 (toxic to Lepidoptera) and Tm 13-14 (toxic to Lepidoptera, Diptera and Coleoptera). It was showed that the Tm 13-14 crystal contained two major peptides of molecular weight 138 and 132 kDa and a minor peptide of 65 kDa. Peptide components of the Tm 13-14 crystal protein resistant to the proteinase of the gut juice of three test species differed from those of the other strains and were purified using sephadex G-100 and DEAE-52 chromatographically. Proteinase resistant peptides (PRPs) toxic to the larvae of *B. mori* were found to be of 68.5 and 59 kDa, while those toxic to larvae of *S. litura* were of 71.65 and 59.6 kDa.

Lambert *et al*. (1996) reported the full characterization of a novel insecticidal crystal protein named Cry9 Ca1 according to the revised nomenclature for Cry proteins from *B. thuringiensis* subsp. *tolworthi.* The crystal protein had 1,157 amino acids and a molecular mass of 129.8 kDa. It showed the typical features of the lepidopteran active crystal proteins such as five conserved sequence blocks. The crystal protein had a fairly broad spectrum of activity against lepidopteran insects including members of families Pyralidae, Plutellidae, Sphingidae and Noctuidae.

Liu *et al*. (1998) studied the effects of combination of *B. thuringiensis* spores and toxins on the mortality of diamondback moth (*P. xylostella*) larvae in leaf residue bioassays. Spores of *B. thuringiensis* subsp. *kurstaki* were found to increase the toxicity of crystals of *B. thuringiensis* subsp. *kurstaki* to both resistant and susceptible larvae. Synergism was found to occur between Cry2A and *B.*

thuringiensis subsp. *urstaki* spores against susceptible larvae and between Cry1C and *B. thuringiensis* subsp. *aizawai* spores against resistant and susceptible larvae.

According to Grove *et al*. (2001), proteolytically activated ICPs significantly reduced the life spans of adult *H. virescens* and *Spodoptera exigua* at a concentration of 500 µg per ml. Individually activated ICPs were differentially toxic to adult *H. virescens* and *S. exigua*. In the study, adult *S. exigua* were found to be sensitive to Cry1C protoxin at a concentration of 1mg per ml.

Obeidat *et al*. (2004) investigated *B. thuringiensis* in four different habitats (grain dust, olive cultivated soils, waste and industrial products, contaminated soils and animal bye- product contaminated soils. Out of the 26 isolated strains, (serotype: *kenyae, kurstaki, kurstaki* HD-1, *thuringiensis*) that produced bipyramidal crystal were found to be toxic to the larvae of *E. kuhniella*. The SDS-PAGE protein profile showed a relationship between the crystal protein shape and toxicity to the larvae of the tested insect.

Cabrera *et al*. (2006) performed bioassays with *Sesamia nonagrioides* (Lefebure) to understand the mode of action of Cry1Ab toxin (binding to specific receptors, stability of binding and pore formation) and the mode of action of other Cry proteins (Cry1Ac, Cry1Ca and Cry1Fa) that were found to be active. Binding assays were performed with I^{125} or biotin labelled toxins and larval brush border membrane vesicles (BBMV).

Pardo *et al*. (2006) studied the activity of seven *B. thuringiensis* Cry1 toxins (five wild type and two domain swapped) against the newly hatched larvae of *S. frugiperda* from adults collected from the field using surface contamination procedure. The CryAAC (1Ac/1Ac/1Ca) hybrid showed the lowest effective dose (LD_{50}) of 288 ng per cm^2 contrasting with parent Cry1Ca and Cry1Ac, which were not lethal (LD_{50}>3000). The wild type Cry1Fa was found to be second most effective

toxin (LD50 565 ng/cm^2). Hybrid HO4 (1Ab/1Ab/1Ca) was only weakly toxic (LD_{50} 1750 ng/cm^2). This was the first report where Cry AAC hybrid toxin was tested against the insect pest.

Shanmugam *et al*. (2006) evaluated the different responses of larval instars of *H. armigera* to *B. thuringiensis* ICPs and suitability of bioassay method to study the toxicity. In the study, it was found that the neonates of the test insect were highly susceptible to *B. thuringiensis* ICPs than second and third instars. The median lethal concentrations (LC_{50}) of *B. thuringiensis* ICPs viz. Cry1Aa, Cry1Ab and Cry1Ac to the neonates were 225.2, 346.8 and 101.7 ng/cm^2 and 0.34, 0.69 and 0.24 µl per ml respectively for surface contamination method and diet incorporation method. The LC_{50} for Cry1Aa, Cry1Ab and Cry1Ac were found to be 203.9, 296.2 and 133.5 ng per cm^2 and 267.4, 456.6 and 160.5 ng per cm^2 respectively to the second and third instar larvae by surface contamination method.

Asokan and Puttaswamy (2007) conducted a bioassay with 18 isolates of *B. thuringiensis* isolated from different sources (soils from different agro climatic zones in Karnataka, leaves, seed dust and insect cadaver) that produced bipyramidal crystals. These isolates were found to be toxic to five days old larvae of *P. xylostella*.

2.6 Vegetative Insecticidal Proteins (Vips)

The Vegetative Insecticidal Proteins (Vips) were secreted during the vegetative growth by *B. thuringiensis* without forming crystals (Estruch *et al*., 1996). The binary proteins Vip-1 and Vip-2 were specific towards Coleoptera where as unrelated Vip-3 was active against Lepidoptera. Vip3A were bound to the midgut epithelium of susceptible larvae but not to that of insensitive species, causing cell death in a process resembling apoptosis (Yu *et al*., 1997). Vip3A production by germinating spores was an important factor in a combined toxicity of spores and relatively inactive Cry toxins against several insects (Donovan *et al*., 2001). Their activity to some agronomically important pests that are less susceptible to *B.*

thuringiensis crystal proteins such as the black cut worm, *A. ipsilon* (Lepidoptera: Noctuidae) and differences in the mode of action make them interesting candidates to complement ICPs in resistance management strategies (Lee *et al*., 2003).

2.6.1 Effect of Vips on insects

Estruch *et al*. (1996) isolated a novel vegetative insecticidal gene Vip3(a), whose gene product showed activity against lepidopteran insect larvae including black cut worm (*A. ipsilon*), fall army worm (*S. frugiperda*), beet army worm (*S. exigua*), tobacco bud worm (*H. virescens*) and corn ear worm (*Helicoverpa zea* Boddie) from *B. thuringiensis* strain AB88. The study revealed that unlike the δ- endotoxins whose expression was restricted to sporulation, Vip3A insecticidal proteins were expressed in the vegetative stage of growth starting at midlog phase as well as during sporulation. Vip3A represented a novel class of proteins, insecticidal to lepidopteran larvae.

Selvapandian *et al*. (2001) cloned and sequenced a vegetative insecticidal protein (Vip) encoding gene from a local isolate of *B. thuringiensis*. It was expressed in *Escherichia coli*. The expressed protein showed insecticidal activity against several lepidopteran pests but was ineffective against *A. ipsilon*. Analysis of insecticidal activity with N and C terminus deletion mutants suggested a different mode of action of Vips against different pests.

2.7 Host range of *B. thuringiensis*

The infection of *B. thuringiensis* was first reported in Japan, from silkworm (*B. mori*) by Ishiwata (1901). This led to the discovery of *B. thuringiensis.* Later Berliner (1915) discovered the same entomopathogen from Mediterranean flour moth (*E. kuhniella*).

The first practical application of *B. thuringiensis* was reported by Husz (1928), who isolated a *B. thuringiensis* strain from *E. kuhniella* and tested it on

European corn borer, *Ostrinia nubilalis* (Hubner). This work eventually led to the first commercial product, Sporein (containing *B. thuringiensis*) that was produced in France in 1938 (Luthy *et al*., 1982).

A major conceptional broadening in the host range of *B. thuringiensis* came in 1977, with the discovery of *B. thuringiensis* subsp. *israelensis* by Goldberg and Margalit (1977) active against mosquito and blackfly larvae (Diptera). This was the first report of a *B. thuringiensis* strain killing a non lepidopteran target.

In the mean time, the range of *B. thuringiensis* was expanded by the discovery of *B. thuringiensis* strains active against Colarado potato beetle *Leptinotarsa decemlineata* (Say). Krieg *et al*. (1983) isolated a strain (*B. thuringiensis* subsp. *tenebrionis*) from a similar st rain, *B. thuringiensis* subsp. *morrisoni* named *B. thuringiensis* subsp. *sandiego* and sequenced the toxic gene (Herrnstadt *et al*., 1986). Different strains of *B. thuringiensis* were now shown to be toxic to three insect orders.

Today a number of isolates of *B. thuringiensis* are commercially produced with its activity against Lepidoptera, Coleoptera and Diptera. *B. thuringiensis* isolates active against insect orders like Hymenoptera, Hemiptera, Orthoptera and Mallophaga and against nematodes, mites and protozoa were also reported (Feitelson *et al*., 1992; Garcia-Robeles *et al*., 2001).

2.8 Host spectra of *B. thuringiensis*

The bacterium, *B. thuringiensis* was reported to cause infection in several insects. Table 1 gives information about the reported host spectra of the bacterium.

Table 1. Host spectra of *Bacillus thuringiensis*

Sl No.	Common name	Scientific name	References
1	Castor semilooper	*Achaea janata* Linnaeus	Devi *et al*. (2001)
2	Lesser wax moth	*Achroia grisella* (Fabricius)	Burges and Hussey, 1971
3	Leaf-cutter ants	*Acromyrmex* sp.	Pinto *et al*. (2003)
4	Field bean pod borer	*Adisura atkinsoni* Moore	Manimegalai *et al*. (2005)
5	Yellow fever mosquito	*Aedes aegypti* Linnaeus	Poncet *et al*. (1997)
6	Alder leaf beetle	*Agelastica alni* Linnaeus	Sezen *et al*. (2006)
7	Black cut worm	*A. ipsilon* (Hufnagel)	Burges and Hussey, 1971
8	Cotton leaf worm	*Alabama argillacea* (Hubner)	Burges and Hussey, 1971
9	Fall cankerworm	*Alsophila pometaria* (Harris)	Burges and Hussey, 1971
10	Summer Chafer	*Amphimallon solstitiale* Linnaeus	Sezen *et al*. (2006)
11	Peach twig borer	*Anarsia lineatilla* Zeller	Devi *et al*. (2001)
12	Silk worm	*Antheraea pernyi* Guerin-Meneville	Heimpel and Angus, 1963
13	Cupreous chafer	*Anomala cuprea* L.	Ohba *et al*. (1992)
14	Cupreous chafer	*Anomala corpulenta* Motsch.	Yu *et al*. (2006)
15	Mosquito	*Anopheles albimanu* Wiedemann	Ruiz *et al*. (2004)
16	Mosquito	*Anopheles stephensi* Liston	Poncet *et al*. (1997)
17	Soybean beetle	*Anomala rufocuprea* L.	Ohba *et al*. (1992)
18	Velvetbean caterpillar	*Anticarsia gemmatalis* Hubner	Bobrowski *et al*. (2001)
19	Fruit tree leaf roller	*Archips argyrospilus* (Walker)	Burges and Hussey, 1971
21	Olive fruit fly	*Bactrocera oleae* (Gmelin)	Sivropoulou *et al*. (2000)
22	*Biomphalaria* snails	*Biomphalaria alexandrina*	Salem, 2004

23	Silk worm	*Bombyx mori* (Linnaeus)	Heimpel and Angus, 1963
24	Round worm	*Caenorhabditis elegans* Maupas	Marroquin *et al*. (2000)
25	Spruce web spinning sawfly	*Cephacia abietis*	Garcia-Robles *et al*. (2001)
26	Spruce bud worm	*Choristoneura fumiferana* (Clemens)	Burges and Hussey, 1971
27	Alfalfa caterpillar	*Colias eurytheme* Biosdual	Heimpel and Angus, 1963
28	Lawn moth	*Crambus sperryillus* (Walker)	Burges and Hussey, 1971
29	Common house mosquito	*Culex fatigans* Wiedemann	Misra *et al*. (2002)
30	Southern house mosquito	*Culex quinquefasciatus* Say	Poncet *et al*. (1997)
31	Walnut caterpillar	*Datana integerrima* Grote and Robinson	Burges and Hussey, 1971
32	Grape leaf folder	*Desmia funeralis* (Hubner)	Burges and Hussey, 1971
33	Western root worm	*Diabrotica virgifera* LeConte	Moellenbeck *et al*. (2001)
34	Southern corn root worm	*Diabrotica undecimpunctata* Howardi	Rupar *et al*. (1991)
35	Mulberry leaf webber	*Diaphania pulverulentalis* (Hampson)	Manimegalai *et al*. (2005)
36	Root weevil	*Diaprepes abbreviatus* Linnaeus	Weathersbee *et al*. (2006)
37	Sugarcane borer	*Diatraea saccharalis* (Fabricius)	Rosas-Garica *et al*. (2004)
38	Common pine sawfly	*Diprion pini* Linnaeus	Garcia-Robles *et al*. (2001)
39	Mediterranean or Moroccan locust	*Dociostaurus maroccanus* (Thunberg)	Aldebis *et al.* (1994)
40	Linden looper	*Erannis tiliaria* (Harris)	Burges and Hussey, 1971
41	Saltmarsh caterpiller	*Estigmene acrea* (Drury)	Burges and Hussey, 1971

42	Mediterranean flour moth	*Ephestia kuhnilla* (Zeller)	Heimpel and Angus, 1963
43	Mexican bean beetle	*Epilachna varivestis* Mulsant	Pena *et al.* (2006)
44	Tur plume moth	*Exelastis atomosa* (W.)	Puntambekar *et al*. (1997)
45	Greater wax moth	*Galleria mellonella* (Linnaeus)	Heimpel and Angus, 1963
46	Tobacco bud worm	*Heliothis virescens* (F.)	Burges and Hussey, 1971
47	Boll worm	*Heliothis zea* (B.)	Burges and Hussey, 1971
48	American boll worm	*Helicoverpa armigera* (H.)	Burges and Hussey, 1971
49	Horn flies	*Haematobia irritans* (L.)	Temeyer (1984)
50	Western tussock moth	*Hemeracampa vetus* (Boisdual)	Burges and Hussey, 1971
51	Asian cockchafer	*Holotrichia parallela* (Motschulsky)	Yu *et al*. (2006)
52	Fall web worm	*Hyphantria cunea* (Drury)	Burges and Hussey, 1971
53	Coffee berry borer	*Hypothenemus hampei* (Ferrari)	Rosa *et al*. (2005)
54	Cigarette beetle	*Lasioderma serricorne* (Fabricius)	Tsuchiya *et al*. (2002)
55	Colorado potato beetle	*Leptinotarsa decemlineata* (Say)	Rupar *et al*. (1991)
56	Potato aphid	*Macrosiphum euphorbiae* (Thomas)	Walters and English (1995)
57	Eastern tent caterpillar	*Malacosoma americanum* (Fabricius)	Burges and Hussey, 1971
58	Tarnished plant bug	*Lygus hesperus* Knight	Desbiens and Cote (2004)
59	Western tent caterpillar	*Malacosoma californieum* (Packard)	Burges and Hussey, 1971
60	Forest tent caterpillar	*Malacosoma disstria* (Hubner)	Burges and Hussey, 1971
61	Great basin tent caterpillar	*Malacosoma fragile* (Stretch)	Burges and Hussey, 1971

62	Tobacco horn worm	*Manduca sexta* (L.)	Heimpel and Angus, 1963
63	Tomato horn worm	*Manduca quinuequema* (Haworth)	Heimpel and Angus, 1963
64	Root Knot Nematode	*Meloidogyne* sp. (Eisenback)	Rai and Rana (1979)
65	Common cockchafer	*Melolontha melolontha* (L.)	Sezen *et al*. (2006)
66	Brown tail moth	*Nygmia phaearrhoea* (Donovan)	Burges and Hussey, 1971
67	Winter moth	*Operophtera brumata* (Linnaeus)	Burges and Hussey, 1971
68	European corn borer	*Ostrinia nubilalis* (H.)	Burges and Hussey, 1971
69	Chinese rice grass hopper	*Oxya chinensis* (Thunberg)	Ren *et al*. (2002)
70	Spring cankerworm	*Paeacrita vernata* (Peck)	Burges and Hussey, 1971
71	Orangedog	*Papilio cresphon* Cramer	Burges and Hussey, 1971
72	Pink boll worm	*Pectinophora gossypiella* (Saunders)	Burges and Hussey, 1971
73	Woolly bear	*Pericallia ricini* (Fabricius)	Gloriana *et al*. (2000)
74	Large white butterfly	*Pieris brassicae* (L.)	Heimpel and Angus, 1963
75	Imported cabbage worm	*Pieris rapae* (Linnaeus)	Heimpel and Angus, 1963
76	Artichoke plum moth	*Platyptilia carduidactyla* (Riley)	Burges and Hussey, 1971
77	Indian Meal Moth (IMM)	*Plodia interpunctella* H.	Schesser and Bulla (1979)
78	Diamondback moth	*Plutella xylostella* (L.)	Burges and Hussey, 1971
79	Japanese beetle	*Popillia japonica* Newman	Ohba *et al*. (1992)
80	Rough skinned cutworm	*Proxenus mindara* Barnes and Mc Donnough	Burges and Hussey, 1971

81	Army worm	*Pseudaletia unipuncta* (Haworth)	Burges and Hussey, 1971
82	Pink stalk borer	*Sesamia nonagrioides* (L.)	Cabrera *et al*. (2006)
83	Tomato leafminer	*Scrobipalpuloides absolutes* (Meyrick)	Theoduloz *et al*.(1997)
84	Black fly	*Simulium pertinax* Kollar	Cavados *et al*. (2004)
85	Bihar hairy caterpillar	*Spilarctia obliqua* Walker	Mohan *et al*. (2005)
86	Beet army worm	*Spodoptera exigua* (Hubner)	Burges and Hussey, 1971
87	Fall army worm	*Spodoptera frugiperda* (J.E Smith)	Burges and Hussey, 1971
88	Egyptian cottonworm	*Spodoptera littoralis* (B.)	Regev *et al*. (1996)
89	Western yellow stripped army worm	*Spodoptera praefica* (Grote)	Burges and Hussey, 1971
90	Satin moth	*Stilpnotia salicis* (Linnaeus)	Burges and Hussey, 1971
91	Processionary caterpillars	*Thaumetopoea solitari* Frey	Kubilay *et al*. (2007)
92	European skipper	*Thymelicus lineola* (Ochsenheimer)	Burges and Hussey, 1971
93	Bag worm	*Thyrdopteryx ephemeraeformicus* (Haworth)	Burges and Hussey, 1971
94	Webbing clothes moth	*Tineola bisselliella* (Hummer)	Burges and Hussey, 1971
95	Cabbage looper	*Tricoplusia ni* (Huber)	Burges and Hussey, 1971
96	Celery leaftier moth	*Udea rubigalis* (Guenee)	Burges and Hussey, 1971
97	European shot-hole borer	*Xylotrechus dispar* (F.)	Sezen *et al*. (2006)

2.9 Effect of *B. thuringiensis* on insects

2.9.1 Effect on *S. litura*

Laboratory tests conducted by Sareen *et al.* (1983) in Uttar Pradesh determined the effect of different concentrations of *B. thuringiensis* subsp. *thuringiensis* on *S. litura* in which soybean leaves were fed to the larvae. The larval mortality was found to increase with increasing doses and the first instar larvae were more susceptible than the later instars.

Zaz and Kushwaha (1993) determined the pathogenicity of *B. thuringiensis* to six larval instars of *S. litura* reared on cauliflower leaves in the laboratory at 35^0 C and 70 per cent relative humidity (RH). The mortality and deformity decreased from the first instar (87.5 %) to sixth instar (32.5%) and a decrease in mortality was found with decrease in concentration.

Puntambekar *et al*. (1997) conducted a laboratory evaluation of different subspecies of *B. thuringiensis* and found that *kurstaki* at 10^8 spores per ml caused more than 85 per cent mortality to the neonates of *S. litura*.

Gloriana *et al*. (2000) tested the pathogenicity of *B. thuringiensis* subsp. *kurstaki* on different larval stages of *S. litura*. Laboratory studies showed that the early instars were susceptible to *B. thuringiensis*. Higher doses were found to be effective in later instars. The LC_{50} values for *S. litura* ranged from 14.8 to 17.3 log cells per ml for *B. thuringiensis* with variation among instars.

Prabagaran *et al*. (2002) isolated *B. thuringiensis* strains from different agro climatic regions of India. The isolates were found to harbor Cry1 family genes. Out of 831 strains, 18 were found to produce 130 and 68 kDa molecular weight proteins. These isolates were subjected to bioassay against second instar larvae of *S. litura*. Among the strains, PBT-372 was found to be superior to other *B. thuringiensis* strains. The results indicated that PBT-372 possessed multiple c*ry* genes.

Tripathi and Singh (2002) evaluated the bioefficacy of *B. thuringiensis* subsp. *kurstaki* against larvae of *S. litura*. The study showed that early instars recorded higher mortality as compared to later instars. They had concluded that the older larvae were physically stronger due to manifold increase in body weight and much higher doses were needed to kill them.

Maghodia and Vyas (2004) studied the efficacy of native *B. thuringiensis* isolate, GAU CT2 in controlling *S. litura* under laboratory and pot culture conditions. Laboratory bioassays showed that the isolate caused maximum mortality to *S. litura* (70.8%) at 10^9 spores per ml after seven days of treatment.

Rajesh *et al*. (2006) examined the bioefficacy of crude protein of 15 *B. thuringiensis* strains isolated from Western Ghat region (Uttar Kannada district of Karnataka and Palakkad district of Kerala) against the second instar larvae of *S. litura*. The strain SW25 was found most effective against the insect larvae and had overlapping fiducial limits with that of the reference strain HD-1.

2.9.2 Effect of *B. thuringiensis* on other lepidopterans

Schesser and Bulla (1978) described the toxicity of *B. thuringiensis* spores to the tobacco hornworm, *M. sexta*. The mortality of larvae was in relation to spore dry weight. Surface application of 6.8 ng per cm^2 resulted in 85 per cent survival but less than 50 per cent survived at 68.2 ng per cm^2.

Theoduloz *et al*. (1997) studied the relative toxicity of native *B. thuringiensis* isolates against the larvae of *S. absolutes*, a South American moth. Four isolates were selected and relative toxicities were evaluated by LD_{50} values. The entomocidal activity of isolate 121e, an autoagglutinating strain, was three fold higher than the toxin synthesized by *B. thuringiensis* subsp. *kurstaki*.

Puntambekar *et al*. (1997) conducted a laboratory evaluation of different *B. thuringiensis* subspecies in which he found that *B. thuringiensis* subsp. *kurstaki* (NCIM 2514) at 10^8 spores per ml caused more mortality to the neonate larvae of *Phthorimaea operculella* (Z.). The strain at 10^{10} and 10^8 spores per ml concentration was found to be effective against major lepidopteran pests comprising the pod borer complex of pigeon pea (*Cajanus cajan*) *viz*., *H. armigera* and *Exelastis atomosa* (W.) under field trials.

Pathogenicity of the entomopathogenic bacterium, *B. thuringiensis* tested on different larval stages of *Pericallia ricini* (F.) by Gloriana *et al*. (2000) showed that the early instars of the pest were susceptible to *B. thuringiensis*. Higher doses of the pathogen were found to be effective on later instars and the LC_{50} values ranged from 10.1 to 18.8 log cells per ml for *B. thuringiensis* with variation among instars.

Porcar *et al*. (2000) tested the insecticidal activity of *B. thuringiensis* against *S. exigua* and two other species of the same genus, *S. littoralis* (B) and *S. frugiperda*. The result indicated that serovars *aizawai*, *thuringiensis* and *kurstaki* were the most frequent within *S. exigua* active strains and that serovar *aizawai* had the highest number of strains exhibiting toxicity against the three species bioassayed.

Polanczyk *et al*. (2000) tested the strains *B. thuringiensis dendrolimus* HD 37, *B. thuringiensis aizawai* HD 68, *B. thuringiensis kurstaki* HD 73, *B. thuringiensis darmstadiensis* HD 146 and *B. thuringiensis thuringiensis* 4412 *in vivo* against second instar larvae of *S. frugiperda* in invivo assays. Suspensions of *B. thuringiensis aizawai* HD 68 and *B. thuringiensis thuringiensis* 4412, containing 3×10^8 spores per ml induced mortality of 100 per cent and 80.4 per cent respectively. To test virulence, cell concentrations of 8×10^5 to 3×10^8 cells per ml of strains of *B. thuringiensis aizawai* HD 68 and *B. thuringiensis* subsp. *thuringiensis* 4412 were applied on the second instar larvae. LC_{50} values were 6.7×10^6 and 8.6×10^6 cells per ml respectively.

Lee *et al*. (2001) isolated a strain of *B. thuringiensis* with dual toxicity from Korean soil samples and named K2. K2 was determined as *B. thuringiensis* subsp. *kurstaki* (H3a3b3c) by serological test and produced bipyramidal parasporal inclusions. Bt K2 was observed to have high toxicity against *S. exigua*.

Bobrowski *et al*. (2001) tested the pathogenicity of 12 *B. thuringiensis* isolates against *Anticarsia gemmatalis* H. Spore–crystal complex was applied to the surface of artificial diet and the mortality was assessed seven days after treatment. When compared to control (*B. thuringiensis* isolate known by its high toxicity to *A. gemmatalis*), four novel *B. thuringiensis* isolates (U87-2, U98-1, U98-4, IP01) exhibited even higher toxic activities against the insect resulting in more than 90 per cent mortality.

Devi *et al*. (2001) conducted bioassays with 22 strains of *B. thuringiensis* belonging to subsp. *thuringiensis*, *kurstaki*, *aizawai*, *galleriae* and *kenyae* against castor semilooper, *Achaea janata* L. Out of these 5 strains, *viz*., 4D6, 4D17, 4D21, 4F2 and AJ4 were found to be potent. Mortality of the neonate larvae ranged from 83 to 100 per cent within two days after treatment even at a low dose of 50 µg *B. thuringiensis* per leaf disc (spore+ toxin). At dose of 500 µg per leaf disc, 12 isolates gave mortalities above 80 per cent at 48 h. after treatment where as 18 isolates gave cumulative mortalities above 90 per cent 5 days after treatment.

Bobrowski *et al*. (2002) characterized two novel *B. thuringiensis* strains isolated from the soils of South Brazil (named UNI 498 and UNI 872) and determined the median lethal concentrations (LC_{50}) of their crystal proteins to *A. gemmatalis* larvae. Seroyping of the new isolates were performed and the results indicated that UNI 872 was a *B. thuringiensis* serovar *kurstaki* and UNI 498 was a *B. thuringiensis* serovar *aizawai*.

Ben-Dov *et al*. (2003) conducted bioassay to find the susceptibility of *B. thuringiensis* subsp. *kenyae* to *S. littoralis*. Reduced mortality was attributed due to feeding inhibition and dilution of pathogen in the presence of nutritional and inert particles, which limited the amount of ingested toxin. The values of LC_{50} of *B. thuringiensis* subsp. *kenyae* against *S. littoralis* were 2.3 to 44 fold higher in the presence of nutritionally or biologically inert (non-nutritional) particles.

Valicente and Barreto (2003) collected a total of 3408 strains of *B. thuringiensis* from 1448 soil samples in 10 Brazilian states, four different geographical regions, covering 96 counties. These strains were evaluated against *S. frugiperda* larvae. Only 62 killed with a mortality between 81 per cent to 100 per cent and 1758 caused no mortality.

Polanczyk *et al*. (2003) tested the susceptibility of *S. frugiperda* against 58 isolates of *B. thuringiensis.* Isolates were applied to the artificial diet at a concentration of 3×10^7 cells per ml against 20 larvae (second instar). Mortality of larvae was taken upto seven days after treatment. *B. thuringiensis* subsp. *morrisoni* isolates were found to be the most pathogenic with 80 per cent mortality. Other seven isolates caused mortality between 40 and 15 per cent, three isolates below 15 per cent and all the others were not effective against the pest. In the virulence assay, *B. thuringiensis* subsp. *morrisoni* showed a LC_{50} value of 8.6×10^6 cells per ml.

Rosas-Garica *et al*. (2004) conducted bioassay studies against sugarcane borer (*Diatraea saccharalis* F.), with *B. thuringiensis* strains HD-133, HD-551, GM-7, GM-10 and GM-34. The strain, GM-34 caused more than 50 per cent mortality with a crystal complex concentration of 50 µg per ml. HD-133 and GM-10 harbored c*ry1C* gene. HD-551 and GM-7 strains harbored c*ry1B* gene while GM 34 strain did not contain Cry1B or Cry1C. All the toxic strains were found to harbor at least one c*ry1A* type gene.

Maghodia and Vyas (2004) studied the efficacy of native *B. thuringiensis* isolate, GAU CT2 in controlling *A. janata* under laboratory and pot culture conditions. Laboratory assays showed that the isolate caused maximum mortality (66.4 %) at 10^9 spores per ml after seven days of treatment.

Asalmol *et al*. (2004) studied the biopesticidal activity of *B. thuringiensis* strains against *H. armigera* in cotton. Among the 17 isolates studied only 10 isolates were pathogenic at 50 µl (toxin) dose and 100 µl (toxin) dose. Isolate Am-II obtained from the local soils of Achalpur in Amaravati district showed good larvicidal effects at both concentrations and the results were superior to standard 4D-1 strain.

Mohan *et al*. (2005) studied the toxicity and growth inhibitory effect of *B. thuringiensis* subsp. *tolworthi* against five important lepidopterous pests in Kumaon hills of Uttaranchal. The neonates of cabbage butterfly, *P. brassicae* and Bihar hairy caterpillar, *S. obliqua* were found highly susceptible (LC_{50} of 0.64 and 2.39 µg/ml respectively) followed by the tomato fruit borer, *H. armigera* and tobacco caterpillar, *S. litura*. The Diamondback moth, *P. xylostella* was completely insensitive to *B. thuringiensis* subsp. *tolworthi* even at high test concentration. The larval growth of third instar *S. litura* and *H. armigera* was severely inhibited with poor growth rates in all treatments.

Manimegalai *et al*. (2005) isolated two new *B. thuringiensis* isolates, *viz*., 01-TAD-01 belonging to *B. thuringiensis* subsp. *sotto* and 01-CHI-01 of *B. thuringiensis* subsp. *kurstaki* from cadavers of silkworm, *B. mori*. Both the strains were found to be pathogenic to mulberry leaf webber, *Diaphania pulverulentalis* (H) causing a mortality of 95 and 78.8 per cent respectively. The isolates 01-CHI-01 and 01-TAD-01 caused a mortality of 91 per cent and 48 per cent for *P. xylostella* and 64 per cent each against *Adisura atkinsoni* M. The isolate caused 85 per cent mortality to *H. armigera.*

Rajesh *et al*. (2006) tested the bio efficacy of crude protein of 15 *B. thuringiensis* strains isolated from Western Ghat region against the second instar larvae of *H. armigera.* Among the various strains tested, SW 25 was found to be most effective and had overlapping fiducial limits with that of the reference strain, HD-1. Polymerase Chain Reaction (PCR) revealed the presence of *cry 1Ac* gene in SW 25.

Kaur *et al*. (2006) characterized 11 isolates of *B. thuringiensis* morphologically and biochemically and evaluated the insecticidal activity against *P. xylostella* under field cum laboratory conditions. Results of bioassay studies revealed that four isolates, namely, Bt 5, 4D4, Bt9 and MTCC 868 caused complete mortality to the second instar larvae of *P. xylostella* within 96 h. at uniform concentration of 10^9 spores per ml.

Kalia *et al*. (2006) studied the temporal susceptibility of neonates of the American boll worm, *H. armigera* to *B. thuringiensis* subsp. *kurstaki* HD-73 or its Cry1Ac toxin in the artificial diet assays for the populations from four different locations. The insect populations, collected post- *Bt* cotton introduction, showed less susceptibility to Cry1Ac/ HD-73 in the artificial diet assays. The larval susceptibility to Cry1Ac in the cotton leaf dip assays was higher than in the artificial diet assays, possibly due to the synergism of Cry1Ac with cotton leaf allelochemicals.

Gitahy *et al*. (2007) selected and characterized a Brazilian *B. thuringiensis* strain with prominent activity towards *D. saccharalis*. Strain S-76 was 11 fold more active than the HD-1 Lepidoptera standard strain. The plasmid profile of strain S-76 was similar to that of HD-1.

Kubilay *et al.* (2007) conducted a study to determine the effect of *B. thuringiensis* subsp. *kurstaki* on the larvae of *Thaumetopoea solitari* F. Four larval instars were tested with various concentrations of the bacterium by dipping Pistachio saplings in relevant suspensions and larvae were fed on the leaves. The effect of *B.*

thuringiensis subsp. *kurstaki* was significantly higher to the first instar larvae than to the second and third instar larvae. Similarly the effect was significantly higher on the second instar larvae than on the third instar larvae.

2.9.3 Effect of *B. thuringiensis* on other insects

2.9.3.1 Effect on Coleoptera

Rupar *et al*. (1991) isolated two novel strains of *B. thuringiensis* from native habitats by the use of genes coding for proteins toxic to coleopterans (*cryIII* genes) as hybridization probes. Strain EG2838 and strain EG4961 contained a CryIIIA-hybridizing plasmid of approximately 95 mDa and synthesized crystal proteins of 74, 70, and 30 kDa. Structural relationships among the crystal proteins of strains EG2838 and EG4961 were detected. Experiments with *B. thuringiensis* flagella antibody reagents demonstrated that EG2838 belonged to H serotype 9 (reference strain *B. thuringiensis* subsp. *tolworthi*) and that EG4961 belonged to H serotype 18 (reference strain B. *thuringiensis* subsp. *kumamotoensis*). A mixture of spores plus crystal proteins of either EG2838 or EG4961 was toxic to the larvae of Colorado potato beetle, *Leptinotarsa decemlineata* (S.) and significantly, the EG4961 mixture was also toxic to the larvae of southern corn rootworm, *Diabrotica undecimpunctata* H.

Donovan *et al*. (1992) observed that *B. thuringiensis* strains EG2838 and EG4961 were highly toxic to Colorado potato beetle larvae, and only the strain EG4961 was toxic to southern corn rootworm larvae. *CryIII*-type genes toxic to coleopterans were cloned from each strain to investigate the cause of the different insecticidal activities of EG2838 and EG4961. Although the CryIIIB and CryIIIB2 proteins were similar in sequence, they displayed distinct insecticidal activities: CryIIIB was one-third as toxic as CryIIIB2 to Colorado potato beetle larvae, and CryIIIB2, but not CryIIIB, was toxic to southern corn rootworm larvae.

Ohba *et al*. (1992) isolated *B. thuringiensis* serovar *japonensis* from the soil of Japan (flagellar antigen 23). The isolate exhibited high larvicidal activity against coleopterous scarabaeid beetles (cupreous chafer, *Anomala cuprea* L.; the soybean beetle, *Anomala rufocuprea* L., and the Japanese beetle, *Popillia japonica* Newman). No toxicity was shown by this isolate against larvae of Lepidoptera, Diptera, Orthoptera, and adults of a chrysomelid coleopteran.

Bradley *et al*. (1995) studied an uncharacterized strain of *B. thuringiensis* (BtS2), from China. It was observed to carry several crystal protein genes and to be toxic to a wide variety of insects, including some coleopterans. The coleopteran toxicity was traced to a CryIB-class protein. It was found to be toxic to at least two species of coleopteran larvae under certain conditions. In contrast to CryIB toxicity toward lepidopterans, the coleopteran activity of CryIB is enhanced by solubilization and by truncation with trypsin prior to administration.

Tsuchiya *et al*. (2002) examined a total of 2652 Japanese isolates of *B. thuringiensis*, belonging to at least 54 H serogroups, for assessment of the toxicity against the cigarette beetle, *Lasidoderma serricorne* (F) (Coleoptera: Anobiidae). Strong larvicidal activities were associated with 28 isolates (1.1%) when tested with spore/parasporal inclusion mixtures. Serologically, these toxic isolates fell into 4 known H serovars: *thuringiensis* (9 isolates), *kurstaki* (2), *kenyae* (2), and *darmstadiensis* (15). Purified parasporal inclusions of the 10 selected isolates exhibited no larvicidal activity, while the supernatants of liquid cultures showed larvicidal and/or growth inhibitory effects. The activities were fully retained after heat treatment at 100^0 C for 10 min. Overall results suggested that β-exotoxin (or thuringiensin) related substances were responsible for the toxicity of the present *B. thuringiensis* isolates against the cigarette beetle.

Rosa *et al*. (2005) determined the effect of 61 strains of *B. thuringiensis* native to Mexico under laboratory conditions, on the coffee berry borer,

Hypothenemus hampei (F.) (Coleoptera: Curculionidae) through bioassays carried out under controlled conditions (27±2ºC; 75±5% RH; photoperiod 12:12 h L: D). The most susceptible life stage of *H. hampei* proved to be the first instar larva, with an average mean lethal time of 6.4±1.8 days. The most virulent strains to the coffee berry borer, were LBIT-129, LBIT-30 and LBIT-130 with estimated mean lethal concentration values of 21.04, 21.26 and 29.07 µg per g of diet, respectively.

Weathersbee *et al*. (2006) studied a collection of *B. thuringiensis* isolates possibly active against coleopteran insects. Each isolate was cultured, spores and δ- endotoxin crystals were pelleted by centrifugation and lyophilized, and the resulting product was incorporated in insect diet for testing against *Diaprepes abbreviatus* L. neonates. Of 19 *B. thuringiensis* isolates screened for activity against *D. abbreviatus* with a discriminating dose of 250 ppm spores and δ-endotoxin on diet, five were selected for further evaluation in dose-response experiments. The most active isolates were those that expressed *Cry* ET33 and *Cry* ET34, or *Cyt* 2Ca1 proteins. A wild-type *B. thuringiensis* strain that expressed *Cyt 2Ca1* generated the lowest LC_{50} value (50.7 µg/ml).

Yu *et al*. (2006) isolated a new *B. thuringiensis* strain, Bt185, from HeBei soil samples in China. Strain produced spherical parasporal inclusions similar to that of the *B. thuringiensis* subsp. *japonensis* Buibui strain, which showed toxicity to both *Anomala corpulenta* M. and *Popillia japonica* N. PCR–restriction fragment length polymorphism results showed that a novel *cry8*-type gene sequence was found in the Bt185 strain. An additional novel *cry8*-type gene was isolated, having a partial sequence of 2340 bp and encoding a protein of 780 amino acids. However, Bt185 exhibited toxicity against larvae of the Asian cockchafer, *Holotrichia parallela* (M).

Sezen *et al*. (2006) conducted a study to find and identify the more toxic *B. thuringiensis* strains against coleopteran pests. They isolated a *B. thuringiensis* strain (Xd3) from European shot-hole borer, *Xylotrechus dispar* (F.) (Coleoptera:

Scolytidae), a highly damaging pest of hazelnut. Based on various morphological, physiological, biochemical, and molecular characteristics, the bacterial isolate was identified as *B. thuringiensis* subsp. *tenebrionis* (*morrisoni*) serovar H8a8b. This isolate was compared with the reference strains by scanning electron microscopy, SDS-PAGE analysis, *cry* gene content, and insecticidal activity. PCR analysis showed that the Xd3 has a *cry* gene, *cry3*. Toxicity tests were performed against coleopteran species. Cent per cent mortality was observed against larvae of *Agelastica alni* L. (Coleoptera: Chrysomelidae). The others were 90 per cent for *Amphimallon solstitiale* L. (Coleoptera: Scarabaeidae), and *Melolontha. melolontha* (L.) (Coleoptera: Scarabaeidae). The results indicated that *B. thuringiensis* subsp. *tenebrionis* (Xd3) might be valuable as biological control agent for coleopteran insects.

Pena *et al.* (2006) identified an S-layer protein by the screening of *B. thuringiensis* strains for activity against the coleopteran pest Mexican bean beetle, *Epilachna varivestis* M. (Coleoptera: Coccinellidae). They screened two *B. thuringiensis* strain collections containing unidentified Cry proteins and also strains isolated from dead insects. Some of the *B. thuringiensis* strains assayed against *E. varivestis* showed moderate toxicity. A *B. thuringiensis* strain (GP1) isolated from a dead insect producing the parasporal crystal was purified and it showed to have insecticidal activity against *E. varivestis.* The gene encoding this protein was cloned and sequenced. The result showed that S-layer protein was directly involved in toxicity to a coleopteran pest

Kati *et al*. (2007) identified a bacterial isolate (Mm2) (*B. thuringiensis* subsp. *tenebrionis*) of Common Cockchafer, *M. melolontha.* PCR analysis showed that the isolate had *cry3* gene. Toxicity tests showed 80 per cent insecticidal activity against the larvae of *M. melolontha*.

2.9.3.2 Effect of *B. thuringiensis* on Dipteran insects

Temeyer (1984) found a strain of *B. thuringiensis* subsp. *israelensis* to be larvicidal to horn flies, *Haematobia irritans* (L.) (Diptera:Muscidae). Larvicidal activity was restricted to purified crystals and fraction 3, indicating that δ-endotoxin of *B. thuringiensis* subsp. *israelensis* was active against horn fly larvae. Purified crystals produced mortality during larval feeding stages, but not pupal stages. Fraction 3 produced significant mortality during both larval and pupal stages. The mortality data indicated the presence of at least two dipteran-active toxins.

Widner and Whitely (1990) studied the host range specificity of two highly related crystal protein genes from *B. thuringiensis* subsp. *kurstaki* HD-1, designated CryIIA and CryIIB (previously named CryBl and CryB2, respectively). Their respective gene products were found to be 87 per cent identical but exhibited different toxicity spectra. CryIIA showed toxicity to both mosquito and tobacco hornworm larvae, whereas CryIIB was toxic only to the latter.

Poncet *et al*. (1997) integrated the *B. thuringiensis* subsp. *israelensis cry11Aa1* gene (referred to as *cry11A*), encoding a δ-endotoxin with toxicity against *Culex*, *Aedes*, and *Anopheles* larvae, either by a single crossover event [strain 2297(pHT5601)], harboring the entire recombinant plasmid] or by two successive crossover events [strain 2297(*cry11A*)]. The level of the Cry11A production in *B. sphaericus* was found to be high. Two crystalline inclusions were produced in strain 2297(pHT5601). Synthesis of the Cry11A toxin conferred toxicity to the recombinant strains against *A. aegyptii* larvae, for which the parental strain was not toxic. The level of larvicidal activity of strain 2297(pHT5601) against *Anopheles stephensi* L. was as high as that of *B. thuringiensis* subsp. *israelensis.* This suggested a synergy between the *B. thuringiensis* and *B. sphaericus* toxins. The toxicities of parental and recombinant *B. sphaericus* strains against *Culex quinquefasciatus* S. were similar. But the larvae were killed more rapidly by the recombinant strains.

According to Sivropoulou *et al*. (2000), the crystals of the soil-isolated *B. thuringiensis* strain A4 consisted of two polypeptides with molecular mass of 140 kDa and 32 kDa that exhibited insecticidal activity against adult flies of *Bactrocera oleae* (Gmelin).

Misra *et al*. (2002) cloned a 1.9-kb DNA fragment, PCR-amplified from HD549 using c*ryII*-gene-specific primers, and expressed in *E. coli*. The recombinant protein caused lower mortality against third-instar larvae of dipteran insects, *C. fatigans* Wiedemann, *A. stephensi* and *A. aegyptii*. The sequence of the cloned crystal protein gene showed almost complete homology with a mosquitocidal toxin gene from *B. thuringiensis* subsp. *kurstaki*, with only five mutations scattered in different regions.

According to Ruiz *et al*. (2004) *B. thuringiensis* subsp. *medellin* produced numerous proteins among which 94 kDa known as Cry11Bb, has mosquitocidal activity. The mode of action of the Cry11 proteins has been described as similar to those of the Cry1 toxins. The study was conducted to investigate the invivo binding of the Cry11Bb toxin to the midgut of the insect species *A. albimanus*, *A. aegypti*, and *C. quinquefasciatus* by immunohistochemical analysis. The Cry11Bb protein was detected on the apical microvilli of the midgut epithelial cells, mostly on the posterior midgut and gastric caeca of the three mosquito species. Additionally, the toxin was detected in the malpighian tubules of *A. albimanus*, *A. aegyptii*, *C. quinquefasciatus*, and in the basal membrane of the epithelial cells of *A. aegypti* midgut. These results confirmed that the primary site of action of the Cry11 toxins is the apical membrane of the midgut epithelial cells of mosquito larvae.

Cavados *et al*. (2004) investigated the effect of *B. thuringiensis* subsp. *israelensis* endotoxins on larvae of the *Simulium pertinax* K. (Diptera: Simuliidae), a common black fly in Brazil, using several concentrations during 4 h. of the serovar *israelensis* strain IPS-82 (LFB-FIOCRUZ 584), serotype H-14 type strain of the

Pasteur Institute, Paris. The most characteristic effects were midgut columnar cell vacuolization, microvilli damages, epithelium cell contents passing into the midgut lumen and finally the cell death.

Cavados *et al*. (2005) isloated eighteen strains of *B. thuringiensis* from *S. pertinax* larvae naturally occurring in rivers of Southeast Brazil with one demonstrating special toxic effects. Simulated field tests against *S. pertinax* larvae showed that the native Brazilian autoagglutinating *B. thuringiensis* (LFB-FIOCRUZ 1035) has an LC_{50} at least 25 times lower than the standard IPS-82 strain. The same bacterial preparation was also tested against *A. aegyptii* larvae in laboratory trials and the LC_{50} values obtained with LFB-FIOCRUZ 1035 were at least three times lower than the one for the IPS 82 strain. The results indicated that the strain was more toxic than the standard *B. thuringiensis* serovar *israelensis* (H14) in the two dipteran species tested.

Manasherob *et al*. (2006) determined the larvicidal activity of *B. thuringiensis* subsp. *israelensis* against dipteran larvae by four major polypeptides of the parasporal crystalline body produced during sporulation. Cyt1Aa showed the lowest toxicity when used alone but was the most synergistic with any of the other proteins. The sequence of the plasmid pBtoxis, which contained all the toxin genes in this subspecies, revealed a new cyt-like coding sequence named cyt1Ca. The gene was PCR-amplified from pBtoxis and cloned in several vectors, allowing high-level expression in *E. coli*. The biological activity of Cyt1Ca was determined. Toxicity against larvae of *A. aegypti* of Cyt1Ca in recombinant *E. coli* cells was compared with that of Cyt1Aa and Cyt2Ba, and the ability of these proteins to enhance the activity of Cry4Aa was assessed.

2.9.3.4 Effect of *B. thuringiensis* on Hymenopteran insects

Hafez *et al*. (1995) studied the interaction between the insect pest, *A. ipsilon*, the parasite, *Apanteles ruficrus* and the pathogen *B. thuringiensis*. *A. ruficrus*

parasitizing on host larvae treated with *B. thuringiensis* showed significant reduction in the egg production, per cent cocoons formed, longevity of either sex and emerged adults. The mortalities caused by β-exotoxin were high among parasitized host larvae more than the unparasitized ones.

Garcia-Robles *et al*. (2001) described the mode of action of a Cry toxin active in the common pine sawfly, *Diprion pini* (L.) (Hymenoptera: Diprionidae). Strain PS86Q3 contained a long bipyramidal crystal composed of five major proteins. The N-terminal sequence showed that the 155 kDa protein corresponded to Cry5B toxin and the other proteins belong to the Cry5A subgroup. PCR analysis indicated the presence of *cry*5Ac and *cry*5Ba genes, suggesting that Cry5A protein should be Cry5Ac. Activation of protoxins with trypsin or with midgut content from *D. pini* and spruce web spinning sawfly, *Cephacia abietis* (Hymenoptera:Pomphiliidae) was found to produce a single 75 kDa toxin that corresponded to Cry5A by N-terminal sequence. It was responsible for the insecticidal activity. Homologous competition experiments with *D. pini* and *C. abietis* brush border membrane vesicles (BBMV) showed that the binding interaction of Cry5A was specific. The results indicated that the hymenopteran specific Cry5A toxin exerts toxicity by a similar mechanism as Cry1 toxins.

Pinto *et al*. (2003) isolated *B. thuringiensis* from ants of two *Acromyrmex* species, to evaluate its pathogenicity towards these ants. Bacterial isolates of *B. thuringiensis* obtained from *Acromyrmex crassispinus* and *Acromyrmex lundi* have been assayed against *A. lundi* in the laboratory. The bioassays were carried out in BOD at 25°C, with a 12 h. photoperiod, until the seventh day after treatment. The data from the *invivo* assays showed a mortality rate higher than 50 per cent in the target population, with the *Bt* HA48 isolate causing 100 per cent of corrected mortality. The results were promising not only regarding allele identification in new isolates, but also for the assays aimed at determining the *Bt* HA48 LC_{50}'s, which can eventually be applied in controlling of *Acromyrmex* leaf-cutting ants.

2.9.3.5 Effect of *B. thuringiensis* on Hemipteran insects

Walters and English (1995) studied the aphidicidal activity of *B. thuringiensis* delta-endotoxins through artificial diet bioassay. Crystalline preparations of CryIIA, CryIIIA and CryIVD solubilized in a slightly alkaline sucrose/amino acid diet clearly imparted toxicity toward adults of potato aphid, *Macrosiphum euphorbiae* (Thomas) (Homoptera: Aphididae) after four to five days of continuous feeding. Spores from an acrystalliferous strain (EG2205) were not toxic by themselves at 7.75×10^5 spores per ml aphid diet, but did restore toxicity to the filtered CryIIIA solution. Therefore, low levels of spores may be very effective in concert with DET for aphicidal activity. Results also clearly demonstrated that a suspension of crystalline CryIIIA alone, without spores, exhibited toxicity. Therefore, DET might be more toxic to the aphids when imbibed as a fine suspension, perhaps indicating the need for slow solubilization into the aphid midgut.

Desbiens and Cote (2004) conducted a study in which whole culture extracts of 94 *B. thuringiensis* strains from 83 serovars were added to an artificial diet and assayed against first and second instars of *Lygus hesperus* K. (Hemiptera: Miridae). A total of five *B. thuringiensis* strains, *B. thuringiensis* subsp. *thuringiensis*, *thuringiensis* exotoxin +, *morrisoni*, *tolworthi*, and *darmstadiensis* generated more than 98 per cent mortality after seven days of incubation. The screening was repeated with 117 alkali-solubilized trypsin-digested *B. thuringiensis* cultures and the same five *B. thuringiensis* strains showed nearly identical results. All five strains produced beta-exotoxin, which exhibited a wide host spectrum activity.

2.9.3.6 Effect of *B. thuringiensis* on Orthoptera

Aldebis *et al.* (1994) isolated many strains of *B. thuringiensis* from nymphs of the Mediterranean or Moroccan locust, *Dociostaurus maroccanus* (T.).

Herna´ndez-Crespo *et al.* (1994) reported about the appreciable mortality caused by several strains of *B. thuringiensis* evaluated against *D. maroccanus*.

Moraga and Alverez (1997) gave demonstration about bacteria present in the hemolymph of nymphs of *D. maroccanus* when fed with food contaminated by a strain of *B. thuringiensis* subsp. *mexicanensis.*

2.9.4 Effect of *B. thuringiensis* on non-insect pests

2.9.4.1 Effect on Nematodes

Rai and Rana (1979) studied the effect of β-exotoxin of *Bacillus thuringiensis* subsp. *thuringiensis* on *Meloidogyne* sp. from brinjal (*Solanum melongena* L.) var. pusa purple long, and to use certain natural substrates for the growth of this bacterial culture.

Marroquin *et al*. (2000) studied *B. thuringiensis* toxin action and resistance in *Caenorhabditis elegans* M. Extensive damage was caused to the gut when the nematode was fed with *B. thuringiensis* toxin. In addition, a decrease in fertility and death (consistent with toxin effects in insects) were also noticed.

Wei *et al.* (2003) expressed seven different crystal toxin proteins from two largely unstudied *B. thuringiensis* crystal protein subfamilies. Four of these crystal proteins were found to be active against multiple nematode species. Each nematode species tested was susceptible to at least one toxin. A rat intestinal nematode was observed to be susceptible to some of the nematicidal crystal proteins, indicating these may hold promise in controlling vertebrate-parasitic nematodes. Toxicity in nematodes correlates with damage to the intestine, consistent with the mechanism of crystal toxin action in insects. The results demonstrated that at least two *B. thuringiensis* crystal protein subfamilies contain nematicidal toxins.

2.9.4.2 Effect of *B. thuringiensis* on snails

Salem (2004) isolated a new *B. thuringiensis* isolate (designated as 66) from dead *Biomphalaria alexandrina* snails in Egypt.

2.10 Molecular characterization of *B. thuringiensis*

The Polymerase Chain Reaction – Random Amplified Polymorphic DNAs (RAPD-PCR) have been used for genetic and molecular studies, as it is a simple and rapid method for determining genetic diversity and similarity in various organisms. The advantage with this method was that no prior knowledge of the genome under research was necessary (Yoon and Kim, 2001). RAPD and PCR were used to amplify certain segments of a genome by using a short arbitrary primer (Welsh and McCleland, 1990). This method is a means of creating a genetic fingerprint of an organism.

Hansen *et al*. (1998) characterized *B. thuringiensis* isolates from phylloplane of organically cultivated cabbage using molecular and phenotypic methods. The RAPD analysis showed that isolates otherwise indistinguishable could be distinguished by this method. They have found that when colony hybridization data was obtained with RAPD analysis, isolates could be grouped based on genetic potential and DNA fingerprint.

Malkawi *et al*. (1999) recovered sixteen isolates of *B. thuringiensis* from different Jordanian habitats using random amplification of polymorphic DNA (RAPD). Total genomic DNA from each isolate and three reference strains were amplified using decamer primers. Electrophoretic analysis of the amplification products revealed the incidence of polymorphism among the isolates. Dendrogram was constructed by pair-wise comparisons of polymorphic products. The analysis showed some regional variation among the isolates, but did not indicate a clearly defined habitat locational pattern of the DNA polymorphism.

DeAmorim *et al*. (2001) conducted a study for the presence of *B. thuringiensis* subsp. *kurstaki* HD-1 like bacteria in human (nasal swab) and environment (air and water) before and after the aerial application of Foray 48B, which contained *B. thuringiensis* subsp. *kurstaki* HD-1. They used RAPD analysis, cry gene specific PCR and dot blot DNA hybridization technique to screen over 11,000 isolates of bacteria. The random oligonucleotide primers 5'-GTTTCGCTCC-3' and 5'-AAGAGCCCGT-3' (Amersham/Pharmacia Biotech) were chosen for RAPD analysis. These two primers were able to differentiate the strains and the result showed that *B. thuringiensis* subsp. *kurstaki* HD-1 like bacteria were present both in the environment and in the human population after the application of Foray 48B.

Rivera and Priest (2003) allocated 126 strains of *B. thuringiensis* representing 57 serovars to 58 genomic types using RAPD – PCR patterns of the serovars examined. 31 contained at least one strain with a closely related or identical RAPD pattern to a strain from a different serovar.

Pinto and Fiuza (2003) tested the *B. thuringiensis* isolates from soil samples of rice fields in Rio Gande do Sul, Brazil, through PCR aiming to screen six groups of *B. thuringiensis cry* genes which codified active proteins for coleopteran and lepidopteran rice pests.

Gupta *et al*. (2005) characterized 12 *B.thuringiensis* strains, which showed more than 60 per cent mortality against second instar larvae of *H. armigera*, using primers specific for *cryIAb* gene by PCR. PCR analysis of selected strains generated a PCR product profile characteristic of *B.thuringiensis* strains, which were insecticidal to lepidopteran insects.

Sadder *et al*. (2006) analysed the diversity at molecular level of seven Jordanian *B.thuringiensis* isolates for toxicity against important pest using Random

Amplified Polymorphic DNA markers. The total genomic DNA was isolated by Wizard ® genomic DNA purification kit (Promega, USA, part numberA1120). DNA markers showed polymorphism among the isolates tested.

Hendriksen and Hansen (2006) developed a specific procedure for quantification and identification of *B. thuringiensis* subsp. *kurstaki* HD-1 (biopesticide) and to quantify its presence in different kinds of cabbage for human consumption. They found that *B. thuringiensis* subsp. *kurstaki* HD-1 could be distinguished from other *B. thuringiensis* strains by its unique random amplification of polymorphic DNA-PCR pattern with the OPA 9 primer. The results showed that *B. thuringiensis* subsp. *kurstaki* HD-1 from biopesticides could be found in vegetables for human consumption.

Salem *et al*. (2006) evaluated the genetic similarity and phylogenetic relationship among four *B. thuringiensis* subspecies (new isolate and three reference strains). RAPD technique was used in the study. The DNA extraction was done using D Neasy Minikit (Gene Company, Limited, Guangzhou, China). The four isolates were grouped into two major clusters A and B. Cluster A consisted of *B. thuringiensis* (ACCC 10061) strain and Cluster B consisted of three *B. thuringiensis* (66, tt4 and i977) strains.

Materials and Methods

3. MATERIALS AND METHODS

The present study on the "Bio-efficacy and molecular characterization of the native isolates of *Bacillus thuringiensis* Berliner" was conducted in the Department of Agricultural Entomology and Centre for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Vellanikkara, Thrissur during 2006 to 2008. The details of the materials used and the techniques adopted for the investigation are described below.

3.1 Preliminary screening of native isolates of *B. thuringiensis* against *Spodoptera litura*

3.1.1 Rearing of *S. litura*

The larvae of *S. litura* collected from banana fields of College of Horticulture, Thrissur were used for mass rearing in the laboratory. They were reared in the natural diet on castor leaves as well as in semi- synthetic diet (Ballal, 2004) (Plate-1 and Plate-2). Castor leaves were kept in plastic jars of capacity 1.5 l and larvae were released @ 20 larvae per jar. The mouth of the jars was closed with muslin cloth to prevent the escape of larvae. Castor leaves were removed, containers were cleaned daily and fresh leaves were given. Pupae, collected from the jars were kept in folds of soft tissue paper and were placed inside the plastic jar for adult emergence.

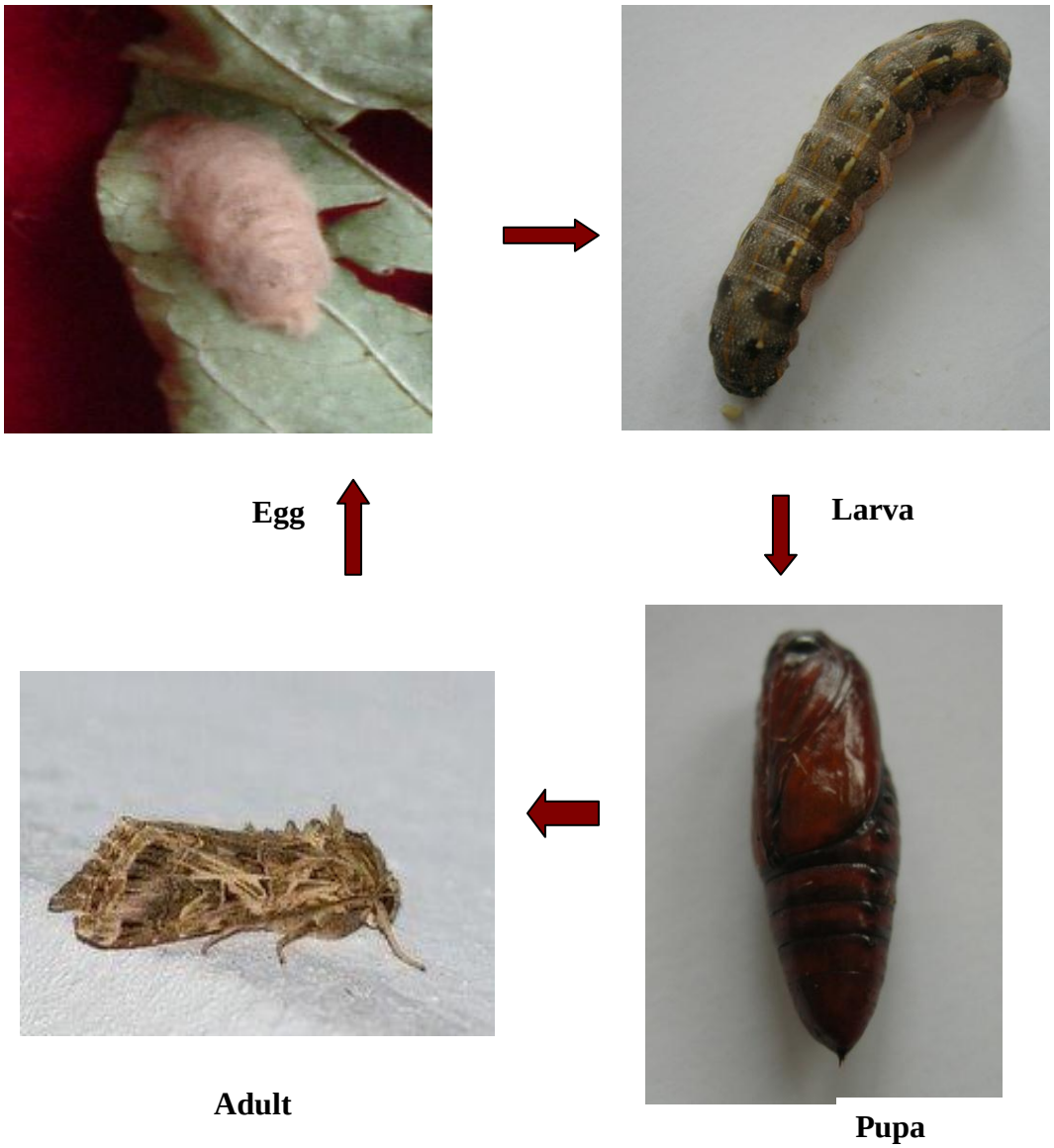
Semi-synthetic diet was used for mass production of test insect so as to get disease free larvae especially during rainy days. Twenty five milliliters of artificial diet was dispensed into sterilized glass beakers of 100 ml capacity and was kept undisturbed until the diet got solidified. Composition of the diet is given in Annexure I. After solidification, the third instar larvae were released @ two larvae per beaker (Mani and Rao, 1998). The beakers were also closed with muslin cloth. The diet was changed at an interval of three days for early instar

Plate – 1 Rearing of *Spodoptera litura* in the laboratory

a) Larvae in semi-synthetic diet

b) Larvae in natural diet on castor leaves

Plate- 2 Different stages of *Spodoptera litura*



larvae and daily for later instar larvae. Pupae were collected and adults were reared out as given below.

Adult moths were fed with 10 per cent honey solution mixed with a few drops of vitamin E (per 25 ml solution). Cotton pieces soaked in honey solution were placed on the sides of plastic jars. Folded papers were kept in the jars for egg laying. Egg masses laid were collected and surface sterilized with 0.05 per cent solution of sodium hypochlorite (NaOCl) for five minutes (Kumar and Ballal, 1991) and kept in separate plastic jars after drying. Castor leaves were given for feeding the neonates. The larvae were transferred to the diet in the third instar @ two larvae per beaker.

3.1.2 Maintenance of *B. thuringiensis* isolates

Twenty native isolates of *B. thuringiensis* collected from Western Ghats of Kerala and the standard reference strain, HD-1 (*B. thuringiensis* subsp. *kurstaki*) were obtained from CPBMB, College of Horticulture, Vellanikkara for conducting the present study. The sites of collection of *B. thuringiensis* were given in Fig. 1. The details of isolates used in the study were given in Table 2.

B. thuringiensis isolates obtained from CPBMB were subcultured in petridishes (90 mm) containing Luria Bertani Agar (LBA) medium (Miller, 1972) and incubated at room temperature and kept in refrigerator for storage. The isolates were subcultured once in two weeks. They were also maintained in T3 Agar medium (Travers *et al*., 1987). Compositions of media were given in Annexure I.

3.1.3 Preparation of spore suspension

B. thuringiensis isolates were cultured in T3 Agar medium and incubated for 24 h. (Plate-3). Then these were transferred to 20 ml of T3 broth in conical flasks of 250 ml capacity. The conical flasks containing the medium were

Fig. 1 Sites of collection of *Bacillus thuringiensis* isolates

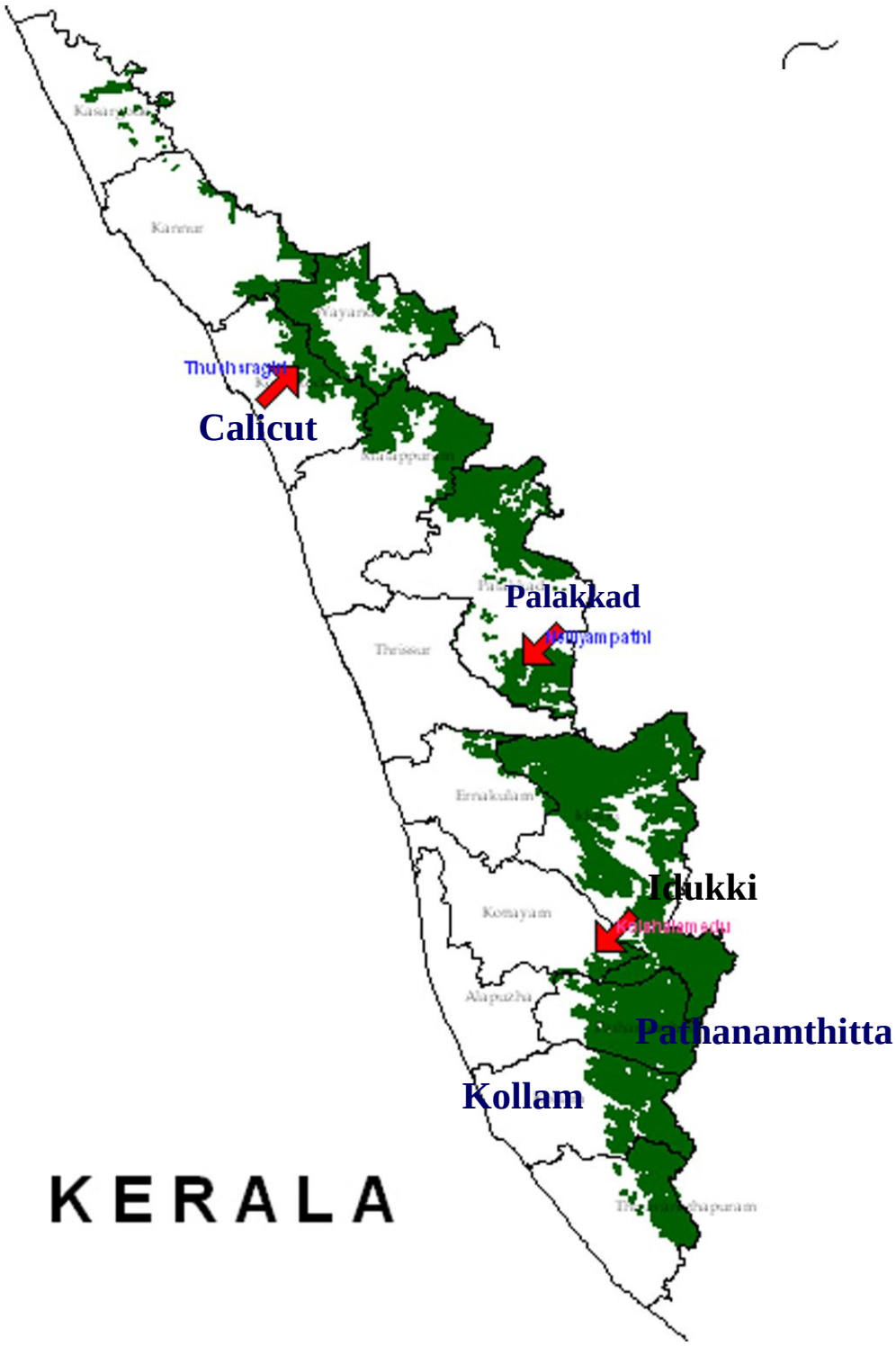
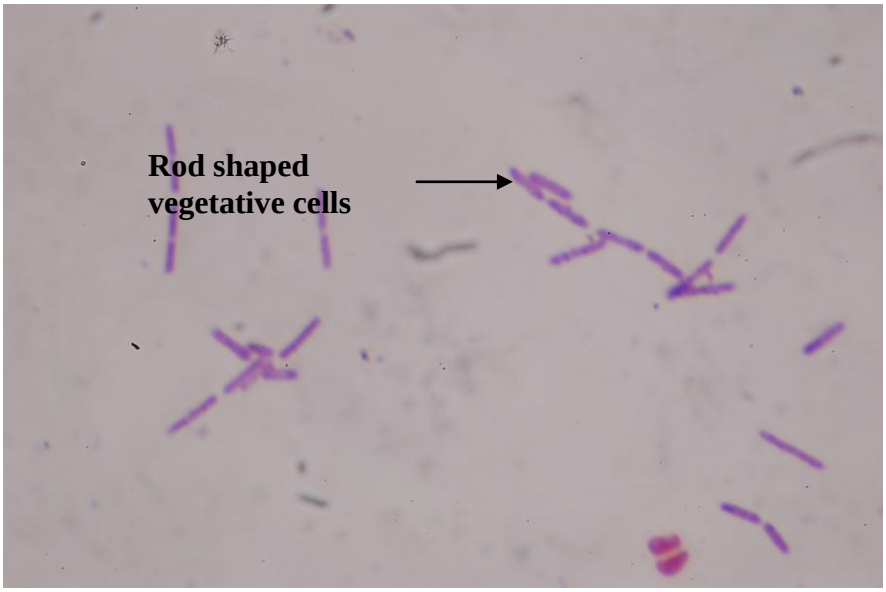


Plate - 3 Rod shaped vegetative cells of *Bacillus thuringiensis*



subjected to shaking (200rpm) for three days in a rotary shaker. The bacterial cultures (liquid medium) were observed daily for sporulation.

Table 2 Details of *Bacillus thuringiensis* isolates used in the study

Sl. No.	**Isolate**	**Crystal protein morphology**	**Location**	**District**	**GPS reading**
1	KAU-1	Spherical	Kolahalamedu	Idukki	N:9° 38.502 E:76° 54.810 Ele: 3608 ft
2	KAU-11	Bipyramidal	Thusharagiri	Calicut	N:11° 31.489 E:75° 49.557 Ele: 340 ft
3	KAU-18	Triangular	Kuttiyadi	Calicut	N:11° 33.242 E:75° 53.593 Ele: 566 ft
4	KAU-19	Irregular + Spherical	Orakkuzhi	Calicut	N:11° 32.654 E:75° 55.513 Ele: 2296 ft
5	KAU-33	Spherical	Kolahalamedu	Idukki	N:9° 38.210 E:76° 55.175 Ele: 3230 ft
6	KAU-37	Irregular	Kolahalamedu	Idukki	N:9° 38.406 E:76° 55.210 Ele: 3210 ft
7	KAU-45	Spherical	Kolahalamedu	Idukki	N:9° 38.724 E:76° 55.213 Ele: 3410 ft
8	KAU-50	Spherical	Kolahalamedu	Idukki	N:9° 38.724 E:76° 55.213 Ele: 3410 ft
9	KAU-51	Bipyramidal	Kolahalamedu	Idukki	N: 9° 38.136 E:76° 42.318 Ele: 989 ft
10	KAU-56	Irregular	Muthappanpuzha	Calicut	N:11° 26.920 E:76° 5.917 Ele: 2014 ft
11	KAU-66	Spherical	Puzhukkanam	Pathanamthitta	N:9° 21.027 E:76° 56.452 Ele: 1479 ft
12	KAU-67	Spherical	Puzhukkanam	Pathanamthitta	N:9° 21.027 E:76° 56.452 Ele: 1479 ft

13	KAU-95	Spherical	Kaduapara	Kollam	N:8° 57.194 E:77° 9.223 Ele: 1055 ft
14	KAU-116	Spherical	Palaruvi	Kollam	N:8° 56.529 E:77° 9.975 Ele: 2002 ft
15	KAU-127	Spherical	Sappal	Palakkad	N:10° 51.920 E: 76° 37.471 Ele: 725 ft
16	KAU-130	Spherical	Dhoni	Palakkad	N:10° 51.834 E:76° 37.217 Ele: 1175 ft
17	KAU-133	Rhomboidal	Dhoni	Palakkad	N:10° 51.775 E:76° 37.238 Ele: 1698 ft
18	KAU-166	Bipyramidal	Nelliyampathi	Palakkad	N:10° 31.097 E:76° 39.603 Ele: 3966 ft
19	KAU-189	Spherical	Nelliyampathi	Palakkad	N:10° 32.138 E:76° 40.080 Ele: 2744 ft
20	KAU-203	Rhomboidal	Nelliyampathi	Palakkad	N:10° 31.373 E:76° 39.206 Ele: 1501ft

N- North; E- East; Ele- Elevation; ft- feet

The spore count per milliliter of broth was calculated by haemocytometer (Lomer and Lomer, 1996), using the following formula,

$$\text{Number of spores per ml} = \frac{X \times 400 \times 10 \times 1000 \times D}{Y}$$

where,

X = Number of spores counted from small squares

Y = Number of smaller squares checked

10 = Depth factor

1000 = Conversion factor from mm^3 to cm^3

D = Dilution factor

Based on spore counting, the concentration of broth was calculated and all the cultures were adjusted to 1 x 10^9 spores per ml (Kaur *et al*., 2006) with 0.1 per cent teepol solution prepared in sterile distilled water. The spore concentration, 1 x 10^9 spores per ml was used as the ideal dosage for treatments since it caused 50 to 80 per cent mortality during the standardization experiments.

For the treatments, the spore suspensions were made directly from the bacterial cultures after dilution. Hence the spore suspension was considered as a spore-crystal complex, containing vegetative insecticidal proteins.

3.1.4 Preparation of leaf discs

Fresh castor leaves were washed thoroughly to remove the dust and dirt and was kept for air drying. After drying, leaf discs of three centimeter (cm) diameter were cut and used for the bioassay.

3.1.5 Bioassay against *S. litura*

Spore suspension containing 1 x 10^9 spores per ml was taken and 100 µl of the suspension was applied uniformly on the upper and lower surface of leaf discs. The same procedure was followed for all the twenty isolates and HD-1. Six leaf discs were used for each isolate.

The spore suspension after pouring on to leaf discs was spread with a glass rod. The discs were kept for 1 to 2 h. for air drying.

After drying, 30 pre-starved (6 h.) early second instar larvae were released to leaf discs @ five larvae per disc. A cotton piece soaked in water was also kept in each petridish to avoid the drying of leaf discs. Fresh leaves were given after the full feeding or complete drying of treated leaf discs. The leaf discs

treated with 0.1 per cent teepol solution alone were kept as control. Observations for mortality were taken at 24 h. interval upto seven days.

The isolates that showed a minimum of 10 per cent mortality was selected for the standardized bioassay.

3.2 Standardized bioassay with the selected isolates

3.2.1 Culturing of *B. thuringiensis* isolates

Bacillus thuringiensis isolates which showed more than 10 per cent mortality against *S. litura* larvae were selected for standardized bioassay. The reference strain, HD-1 was also used in the bioassay to compare the mortality rate. The isolates were cultured in T3 Agar medium in sterile petridishes (Plate- 4). The multiplication of the isolates in T3 broth was done following the procedure described earlier.

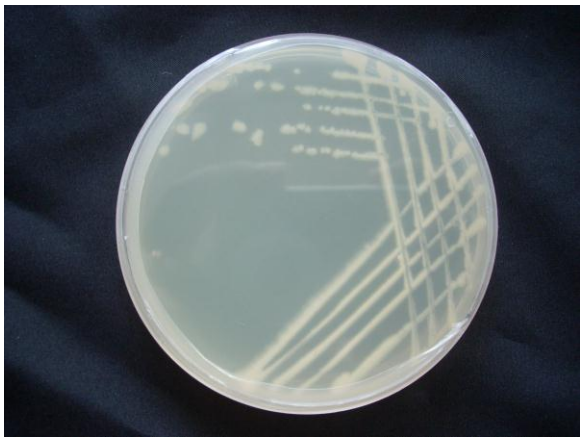
3.2.2 Preparation of spore suspension

The spore counts of all the four isolates were estimated using haemocytometer and spore count per milliliter was calculated. All the spore suspensions were adjusted to 1×10^{11} spores per ml with 0.1 per cent teepol solution. Five different concentrations namely, 1×10^{11}, 1×10^{10}, 1×10^{9}, 1×10^{8}, 1×10^{7} spores per ml were prepared for the bioassay by serial dilution.

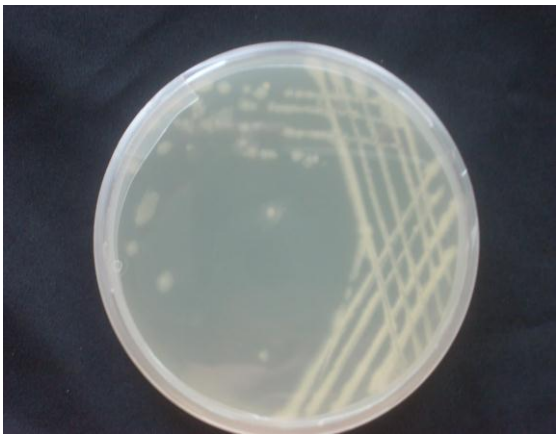
3.2.3 Bioassay against *S. litura*

Fresh castor leaves were cut into small discs of three centimeter diameter. These leaf discs were dipped in spore suspensions of different concentrations (1×10^{11}, 1×10^{10}, 1×10^{9}, 1×10^{8}, 1×10^{7} spores/ml) for 30 seconds @ six leaf discs per each concentration. The leaf discs were kept for air drying and were transferred to petridishes. Cotton pieces soaked in water were kept in petridishes to avoid the drying of leaf discs. Larvae were released into each petridish @ five larvae per petridish. Three replications were kept for each

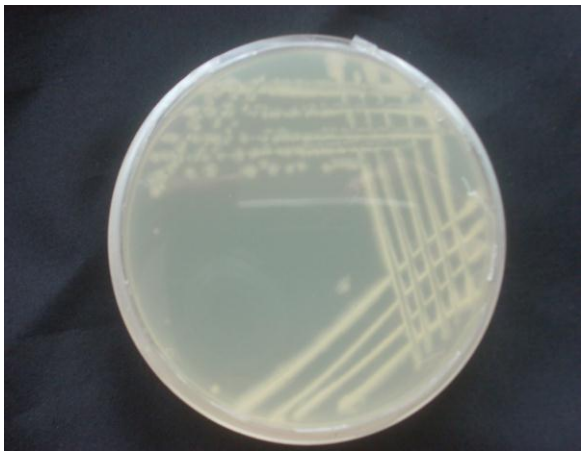
Plate – 4 Selected isolates of *Bacillus thuringiensis*



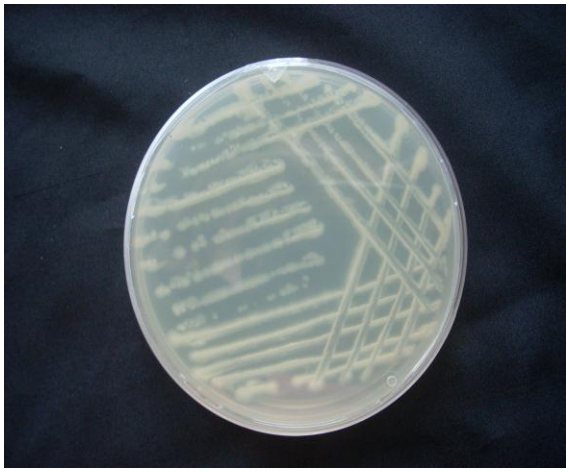
KAU-11 (Thusharagiri)



KAU-51 (Kolahalamedu)



KAU-166 (Nelliyampathi)



HD-1 (Standard Strain)

concentration @ ten larvae per replication. Leaf discs dipped in 0.1 per cent teepol solution alone were kept as control. The experiment was carried out in Completely Randomized Design (CRD). The observations for mortality were recorded upto seven days after treatment. The per cent mortality was analyzed by ANOVA after square root transformation.

3.2.4 Median Lethal Concentration (LC_{50})

The LC_{50} values for the isolates were calculated by Finney's method of probit analysis (Finney, 1971).

3.2.5 Median Lethal Time (LT_{50})

The time required to kill 50 per cent of the test insect used (LT_{50}) was estimated by graphical method.

3.3 Molecular characterization of *B. thuringiensis* isolates

3.3.1 Isolation of total DNA from *B. thuringiensis*

The total DNA was isolated from *B. thuringiensis* isolates and reference strain (HD-1) following the protocol of Sambrook and Russel (2001).

1 25 ml of overnight grown culture of *B. thuringiensis* in LB broth was taken and centrifuged at 10, 000 rpm for 10 minutes at 4°C.
2 Resuspended the pellet in 10 mM Tris Cl and 100 mM NaCl.
3 Centrifuged at 10,000 rpm for 10 minutes at 4°C.
4 The pellet was resuspended in 2.5 ml T_{50} E_{20} and 500 µl of lyzozyme from the stock of 50 mg per ml and incubated at 37 °C for 20 minutes.
5 25 µl of RNAse was added from a stock of 10 mg per ml.
6 Incubated at room temperature for 10 minutes.
7 2.5 ml of SDS *i. e*. two per cent in T_{50} E_{20} was added and incubated at 50°C for 45 minutes.

8 50 µl of proteinase K was added from a stock of 20 mg per ml and incubated at 50- 55 ^{0}C for 10 minutes.

9 An equal volume of phenol was added and mixed gently and centrifuged at 10,000 rpm for 10 minutes at 4 ^{0}C.

10 The aqueous phase was transferred to a fresh tube and added equal volume of phenol: chloroform (1:1) ratio and mixed gently.

11 Centrifuged at 10, 000 rpm for 10 minutes at 4 °C and separated the aqueous phase.

12 An equal volume of chloroform: isoamyl alcohol (24:1) was added and mixed gently.

13 Centrifuged at 10, 000 rpm for 10 minutes at 4^{0}C and separated the aqueous phase.

14 One tenth volume of 3 M Sodium acetate was added and kept in ice for 20 minutes.

15 An equal volume of isopropanol was added and incubated for 5 to10 minutes

16 Centrifuged at 10,000 rpm for 15 minutes at 4°C and decanted the supernatant.

17 The pellet was washed in 70 per cent alcohol, by giving a centrifugation at 10, 000 rpm for three minutes. The supernatant was discarded.

18 The pellet was dried and dissolved in 25 to 30 µl of T_{10} $E_{1.}$

3.3.2 Estimation of quality and quantity of DNA

3.3.2.1 Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed based on the method described by Sambrook *et al*. (1989) to check the quality of DNA and also to separate the amplified products.

Materials

1) Agarose: (Genei, Low EEO)

2) 50X TAE buffer (pH 8.0)

3) Electrophoresis unit, power pack, casting tray, comb.

4) 6X Loading/ Tracking dye

5) Ethidium bromide solution (stock 10 mg per ml; working concentration, 0.5μg per ml)
6) UV transilluminator (Herolab ^{R}, USA)
7) Gel documentation and analysis system (UVP Gel Doc- It ^{TM} Imaging System)

(Chemical composition of the buffer and dyes are given in Annexure III)

3.3.2.1.1 Procedure for casting, loading and running the gel

1 Electrophoresis buffer (1X TAE) was prepared to fill the electrophoresis tank and to prepare the gel

2 The open ends of the gel casting tray were sealed with a cellophane tape and placed on a perfectly horizontal levelled platform.

3 Agarose (0.8 % (w/v) for genomic DNA and 1.2 % for RAPD) was added to 1X TAE, boiled till the agarose dissolved completely and then cooled to lukewarm temperature. Ethidium bromide was added to a final concentration of 0.5 μg per ml as an intercalating dye of DNA, which will help in its visualization in UV spectrum.

4 It was then poured into the gel mould. The comb was placed properly and gel was allowed to solidify.

5 After the gel was completely set (30- 45 minutes at room temperature), the comb and cellophane tape were carefully removed.

6 The gel was placed in the electrophoresis tank with the wells near the cathode and submerged in 1 X TAE to a depth of 1 cm.

7 A piece of cellophane tape was pressed on a solid surface and 1μl 6X loading dye was dispensed in small quantity on the tape. A quantity of three to five μl of DNA was added to each slot, mixed well by pipetting in and out for two to three times. Then the mixture was loaded in the wells, with the help of micropipette. Appropriate molecular weight marker was also added in one of the wells.

8 The cathode and anode were connected to the power pack and the gel was run at a constant voltage of 60 volts.

9 The power was turned off when the tracking dye reached at about three centimeter from the anode end.

3.3.2.1.2 Gel photo documentation

The DNA bands separated by electrophoresis were viewed under UV light in a UV transiluminator and were photographed using UVP Gel Doc- It ^{TM} Imaging System. The DNA fluoresces under UV light due to ethidium bromide dye.

3.3.2.2 Purity of DNA:

The purity of DNA was further analysed by using NanoDrop® ND-1000 spectrophotometer. This is a full spectrum (220-750nm) spectrophotometer that measures 1μl samples with high accuracy and reproducibility.

The absorbance of nucleic acid samples was measured at a wavelength of 260nm and 280nm. The ratio (260/280) was used to assess the purity of nucleic acids.

3.3.3 RAPD- PCR

Genomic variability can be easily assessed based on length and sequence differences in PCR-amplified segments generated with primers of arbitrary nucleotide sequence. RAPD was used as a molecular marker with which the different strains can be characterized. In this technique, a single short oligo nucleotide primer which binds to many different loci is used to amplify random sequences from a template DNA. The amplified DNA samples were electrophoresed as described earlier. The isolated DNA was subjected to RAPD analysis. RAPD programme includes 30 cycles.

One cycle included

a) initial denaturation for 5 minutes at 94° C
b) samples were subjected to 35 cycles of denaturation (94° C, 1 minute)
c) primer annealing (37° C, 1minute)
d) primer extension (72° C, 2 minutes) and
e) final extension of 5 minutes at 72°C

3.3.3.1 Preparation of master mix

The reaction mixture (25µl) consisted of

Taq buffer with $MgCl_2$ (10X)	-	2.5 µl
dNTP mix (10 m M)	-	1.0 µl
Primer (n moles)	-	2.0 µl
Taq DNA polymerase (3U/µl)	-	1.0 µl
Template DNA (50ng/µl)	-	2.0 µl
Distilled water	-	15.5 µl
		25.0 µl

A master mix for the required number of reactions was prepared using the reaction mixture without adding primer and template. From the master mix, 21 µl was pipetted into each PCR tube of 0.2 ml capacity after the addition of 2 µl of primer and 2 µl of template DNA. PCR tubes were loaded in thermal cycler. The programme was run.

The amplified products were electrophoresed on 1.2 per cent agarose gel containing ethidium bromide, using 1X TAE buffer. DNA fragments were viewed under UV light transilluminator and documented using UVP Gel Doc- It ^{TM} Imaging System.

3.3.3.2 Screening of random primers for RAPD

Primer screening was carried out to identify the best primers for RAPD analysis. Twenty randomly selected decamer primers were screened for amplification of genomic DNA extracted from the *B. thuringiensis* isolate, KAU-166 using thermal cycler. Out of the 20 primers, 10 primers which gave good amplification (more than five bands) were selected and utilized for further characterization of the three selected *B. thuringiensis* isolates and the reference strain, HD-1. The total number of amplicons, total number of monomorphic bands and polymorphic bands produced by each primer were recorded. The per cent polymorphism also calculated using the following formula.

$$\text{Per cent polymorphism} = \frac{\text{Total number of polymorphic bands}}{\text{Total number of bands}} \times 100$$

The discrimination power of primers was also calculated using the following formula.

$$\text{Discrimination power of a primer} = \frac{\text{Number of unique RAPD phenotypes}}{\text{Total number of phenotypes}} \times 100$$

The list of primers used for screening in the RAPD analysis is given in Table 3.

Table 3. List of primers used for screening in RAPD

Sl. No	Primers	Nucleotide sequence (5' – 3')	Source
1	OPF 3	CCT GAT CAC C	Operon Technologies,Inc., USA
2	OPF 5	CCG AAT TCC C	Operon Technologies,Inc., USA
3	OPF 11	TTG GTA CCC C	Operon Technologies, Inc., USA
4	OPF 14	TGC TGC AGT A	Operon Technologies, Inc., USA
5	N 6	GAG ACG CAC A	Integrated DNA Technologies (IDT), USA
6	S 01	GTT TCG CTC C	Integrated DNA Technologies (IDT), USA
7	S03	CAT CCC CCT G	Integrated DNA Technologies (IDT), USA
8	S 05	TGC GCC CTT C	Integrated DNA Technologies (IDT), USA
9	S 07	GGT GAC GCA G	Integrated DNA Technologies (IDT), USA
10	S 09	TGG GGG ACT C	Integrated DNA Technologies (IDT), USA
11	S 12	CCT TGA CGC A	Integrated DNA Technologies (IDT), USA
12	S 22	TGC CGA GCT G	Integrated DNA Technologies (IDT), USA
13	S 23	AGT CAG CCA C	Integrated DNA Technologies (IDT), USA
14	S 28	GTG ACG TAG G	Integrated DNA Technologies (IDT), USA

15	S 30	GTG ATC CTG G	Integrated DNA Technologies (IDT), USA
16	S 31	CAA TCG CCG T	Integrated DNA Technologies (IDT), USA
17	S 63	GGG GGT CTT T	Integrated DNA Technologies (IDT), USA
18	S 103	AGA CGT CCA C	Integrated DNA Technologies (IDT), USA
19	S 141	CCC AAG GTC C	Integrated DNA Technologies (IDT), USA
20	S 189	CTG ACG TCA C	Integrated DNA Technologies (IDT), USA

3.3.3.3 Analysis of RAPD profiles

The amplification profiles obtained on RAPD analysis of *B. thuringiensis* isolates were compared and scoring was done as one or zero based on the presence or absence of bands. The data was analysed for the estimation of genetic similarity by computing DICE coefficient using the Numerical Taxonomy System of Multivariate Statistical Programme (NTSyS) software package. The clustering was done and dendrogram was drawn using Unweighted Pair Group Method of Arithematic Averages (UPGMA) by Sneath and Sokal (1973) using the above NTSyS programme.

Results

4. RESULTS

The results of the study on "Bio-efficacy and molecular characterization of the native isolates of *Bacillus thuringiensis* Berliner" conducted during the period 2006 to 2008 at the Department of Agricultural Entomology and at the Centre for Plant Biotechnology and Molecular Biology (CPBMB) were presented below.

4.1 Preliminary screening of the native isolates of *B. thuringiensis* against *Spodoptera litura*

Twenty native isolates of *B. thuringiensis* were screened to find out their pathogenicity to the second instar larvae of *S. litura*. Symptoms developed in the larvae of *S. litura* due to bacteriosis were recorded and the larval mortality was observed.

4.1.1 Syndrome

The larvae showed cessation of feeding as the first symptom after feeding on the treated leaves. Larvae stopped moving and death occurred in 19 to 20 h. Colour of the larvae changed to dark brownish black and the larval body became putrified after death. It changed to black coloured mass. Oozing of brown fluid from the larval body was observed and these contained spores of bacteria. (Plate- 5).

4.1.2 Larval mortality

Results on the larval mortality of the larvae of *S. litura* caused by the 20 isolates of *B. thuringiensis* and the reference strain, HD-1 upto seven days after treatment is given in Table 4.

Plate – 5 Symptoms of *Bacillus thuringiensis* infection

Colour change on the larval body

Apperence of larval body as a black coloured mass

Oozing out of brown fluid from the larval body

Table 4. Larval mortality caused by different isolates of *Bacillus thuringiensis* isolates against larvae of *Spodoptera litura* in the preliminary screening

Sl.No	**Native isolates of *B. thuringiensis* @ 1×10^9 spores per ml**	**Cumulative mortality upto 168 h. (%)**
1	KAU-1	0.0
2	KAU-11	76.7
3	KAU-18	0.0
4	KAU-19	0.0
5	KAU-33	0.0
6	KAU-37	0.0
7	KAU-45	3.0
8	KAU-50	0.0
9	KAU-51	80.0
10	KAU-56	0.0
11	KAU-66	0.0
12	KAU-67	0.0
13	KAU-95	0.0
14	KAU-116	0.0
15	KAU-127	0.0
16	KAU-130	3.0
17	KAU-133	0.0
18	KAU-166	86.7
19	KAU-189	0.0
20	KAU-203	0.0
21	HD-1 (*B.thuringiensis* subsp. *kurstaki*)	96.7

Among the 20 native isolates tested, only five isolates, *viz.*, KAU-11, KAU-45, KAU-51, KAU-130 and KAU-166 caused mortality ranging from 3.00 to 86.7 per cent to *S. litura*. The reference strain, HD-1 showed the highest mortality of 96.7 per cent. Three isolates, KAU-11, KAU-51 and KAU-166 that caused more than 10 per cent mortality along with HD-1 were selected for the standardized bioassay for lepidopteran specificity (Plate-6). Out of the three isolates selected, KAU-166 indicated a highest mortality of 86.7 per cent followed by KAU-51 with 80.0 per cent and KAU-11 with 76.7 per cent.

4.2 Standardized bioassay of the selected lepidopteran specific isolates of *B. thuringiensis*

As the isolates, KAU-11, KAU-51 and KAU-166 were observed to be lepidopteran specific, they were further subjected to standardized bioassay along with HD-1 as standard.

4.2.1 Larval mortality

The per cent mortality obtained in the standardized bioassay with three isolates and HD-1 on the second instar larvae of *S. litura* was given in Table 5.

Plate- 6 Larvae killed by selected isolates of *Bacillus thuringiensis*

KAU-11

KAU - 166

KAU-51

HD-1

Table 5. Mortality of *Spodoptera litura* caused by different concentrations of selected *Bacillus thuringiensis* isolates and HD-1 at different hours after treatment

Isolates	**Concentration (spores/ml)**	**Mortality (%)**						
		24 h.	**48 h.**	**72 h.**	**96 h.**	**120 h.**	**144 h.**	**168 h.**
KAU-11	$1x10^{7}$	10.0	26.7	46.7	53.3	60.0	63.3	76.7
	$1x10^{8}$	6.7	30.0	40.0	50.0	60.0	66.7	76.7
	$1x10^{9}$	3.0	30.0	50.0	60.0	63.3	70.0	80.0
	$1x10^{10}$	3.3	23.3	40.0	66.7	66.7	76.7	83.3
	$1x10^{11}$	16.7	36.7	50.0	63.3	70.0	76.7	90.0
KAU-51	$1x10^{7}$	10.0	26.7	40.0	50.0	60.0	63.3	63.3
	$1x10^{8}$	16.7	33.3	53.3	56.7	60.0	63.3	66.7
	$1x10^{9}$	13.3	43.3	63.3	66.7	73.3	73.3	80.0
	$1x10^{10}$	16.7	40.0	66.7	70.0	73.3	76.7	80.0
	$1x10^{11}$	20.0	50.0	70.0	76.7	76.7	80.0	83.3
KAU-166	$1x10^{7}$	3.3	16.7	40.0	53.3	56.7	60.0	66.7
	$1x10^{8}$	6.7	36.7	53.3	60.0	63.3	66.7	70.0
	$1x10^{9}$	13.3	36.7	56.7	70.0	76.7	80.0	83.3
	$1x10^{10}$	6.7	33.3	70.0	73.3	76.7	80.0	83.3
	$1x10^{11}$	16.7	60.0	73.3	83.3	86.7	93.3	93.3
HD-1	$1x10^{7}$	10.0	30.0	53.3	66.7	66.7	66.7	76.7
	$1x10^{8}$	10.0	40.0	63.3	76.7	83.3	83.3	83.3
	$1x10^{9}$	16.7	50.0	76.7	86.7	86.7	90.0	93.3
	$1x10^{10}$	6.7	56.7	90.0	90.0	93.3	96.7	96.7
	$1x10^{11}$	20.0	53.3	96.7	100.0	100.0	100.0	100.0

The results of the standardized bioassay showed that the per cent mortality varied in different concentrations of bacterial isolates, during different days after treatment. The reference strain, HD-1 caused 100 per cent mortality at a concentration of $1x10^{11}$ spores per ml four days after treatment (DAT) (96 h.). The mortality was found to increase with the increase in concentration. Lowest

mortality (76.7%) was observed in the lowest concentration ($1x10^7$ spores/ml), at 7 DAT. Thus mortality increased from 63.3 per cent in the lower concentration to 100 per cent in the highest concentration.

The isolate, KAU-166 caused the second highest mortality (93.3 %) at the highest concentration ($1x10^{11}$ spores/ ml) at 6 DAT. The mortality was observed to be the same (83.3 %) for the next two concentrations ($1x10^9$ spores/ml and $1x10^{10}$ spores/ml). Lowest mortality (66.7 %) was observed in the lowest concentration ($1x10^7$ spores/ ml).

At 7 DAT, KAU-11 caused 90.0 per cent mortality at the higher concentration of $1x10^{11}$ spores per ml and 76.7 per cent at the lowest concentration.

The highest concentration, $1x10^{11}$ spores per ml caused 83.3 per cent mortality in KAU-51. The mortality rates for $1x10^{10}$, $1x10^9$, $1x10^8$ and $1x10^7$ spores per ml were 80.0 per cent, 80.0 per cent, 66.7 per cent and 63.3 per cent respectively.

Concentration of spores showed a positive effect on the mortality of larvae in the case of all *B. thuringiensis* isolates. The per cent mortality was directly proportional to the concentration of spores used.

An increase in the larval mortality was observed with the prolongation of time. The lowest mortality was observed on the first day (24 h.) in all the isolates and the mortality rate increased with increase of time. Thus the mortality ranged from three to 20.0 per cent in different treatments, 24 h. after treatment to 16.7 per cent to 60.0 per cent on the second day, 40.0 to 96.7 per cent on the third day, 50.0 to 100.0 per cent on the fourth day, 56.7 to 100.0 per cent on the fifth day, 60.0 to 100.0 per cent on the sixth day and 63.3 to 100 per cent on the seventh day. No mortality was recorded in control.

4.2.1.1 Cumulative mortality

The data of cumulative per cent mortality of *S. litura* caused by three selected isolates at seven days after treatment was statistically analysed and the results are presented in Table 6.

Table 6. Cumulative mortality of *Spodoptera litura* in different treatments (7 DAT)

Isolates	**Concentrations (spores/ ml)**				
	$1x10^7$	**$1x10^8$**	**$1x10^9$**	**$1x10^{10}$**	**$1x10^{11}$**
KAU-11	76.7 (8.75)	76.7 (8.75)	80.0 (8.92)	83.3 (9.13)	90.0 (9.48)
KAU-51	63.3 (7.98)	66.7 (8.13)	80.0 (8.93)	80.0 (8.96)	83.3 (9.15)
KAU-166	66.7 (8.17)	70.0 (8.38)	83.3 (9.13)	83.3 (9.14)	93.3 (9.67)
HD-1	76.7 (8.78)	83.3 (9.10)	93.3 (9.68)	96.7 (9.85)	100.0 (10.02)

CD value- NS

* Values in parenthesis are square root transformed values

The results showed that all the isolates were on par in the effectiveness against the larvae of *S. litura*. Treatments under the same concentration showed same effect. No significant difference was noticed between concentrations and between treatments. So all the treatments are equal in effectiveness to the test larvae. No mortality was recorded in control.

4.2.2 Median Lethal Concentration (LC_{50}) of *B. thuringiensis* isolates

The cumulative per cent mortality was subjected to Probit Analysis to determine Median Lethal Concentration (LC_{50}). The LC_{50} for all the isolates with the five different concentrations were estimated by Finney's method of Probit Analysis and was given in Table 7.

Table 7. Median Lethal Concentration (LC_{50}) of selected *Bacillus thuringiensis* isolates

Sl. No.	Isolates	LC_{50} (spores/ml)
1	KAU -11	1.2589×10^6
2	KAU-51	6.3095×10^4
3	KAU-166	7.9432×10^4
4	HD-1	1.5849×10^5

LC_{50} values for the three selected *B. thuringiensis* isolates and HD-1 calculated from the larval mortality caused to early second instar larvae from five different concentrations showed that it varied from 6.3095×10^4 in KAU-51 to 1.258925×10^6 spores per ml in KAU-11. In KAU-166, 50 per cent mortality was recorded at a concentration of 7.9432×10^4 spores per ml and in HD-1, it was 1.5849×10^5.

Lowest LC_{50} was observed in KAU-51 and it showed the efficacy of the isolate in causing 50 per cent death of the insect with the ingestion of relatively less amount of spores.

The isolate, KAU-11 was observed to have the highest LC_{50} value showing the comparatively low effectiveness of the isolate to the test insect.

4.2.2 Median Lethal Time (LT_{50}) of *B. thuringiensis* isolates

Median Lethal Time for different concentrations of *B. thuringiensis* isolates was estimated from the cumulative per cent mortality by graphical method and is given in Table 8.

Table 8. Median Lethal Time (LT_{50}) of different *Bacillus thuringiensis* isolates for different concentrations

Isolates	**Concentrations (spores/ml)**	**LT_{50} (h.after treatment)**
KAU-11	$1x10^7$	80.5
	$1x10^8$	96.0
	$1x10^9$	72.6
	$1x10^{10}$	81.6
	$1x10^{11}$	72.0
KAU-51	$1x10^7$	96.0
	$1x10^8$	66.6
	$1x10^9$	55.2
	$1x10^{10}$	57.0
	$1x10^{11}$	48.0
KAU-166	$1x10^7$	89.3
	$1x10^8$	66.4
	$1x10^9$	63.6
	$1x10^{10}$	59.4
	$1x10^{11}$	56.0
HD-1	$1x10^7$	68.0
	$1x10^8$	57.5
	$1x10^9$	48.0
	$1x10^{10}$	44.4
	$1x10^{11}$	45.4

In the case of HD-1, the highest concentration took 45.4 h. to cause 50 per cent mortality. The values for $1x10^{10}$, $1x10^9$, $1x10^8$ and $1x10^7$ spores per ml concentrations were 44.4 h., 48.0 h., 57.5 h. and 68.0 h. respectively. The LT_{50}

values of HD-1 for all concentrations were less compared to other isolates. This showed that the standard strain used was ranked best based on time response to cause mortality.

The LT_{50} values of the tested five concentrations of KAU-166, ranged from 56.0 to 89.3 h. An increase in LT_{50} values with decrease in concentration was observed.

At a concentration of $1x10^{11}$ spores per ml, the isolate KAU-51 took 48 h. to kill 50 per cent of the treated insects. The lowest concentration ($1x10^{7}$) took 96 h. for 50 per cent mortality.

For the isolate, KAU-11, the LT_{50} value at the higher concentration ($1x10^{11}$ spores/ml) was 72 h. The LT_{50} values for the rest of concentrations ranged from 72 h. to 96 h.

4.3 Molecular characterization of *B. thuringiensis* isolates

The three different *B. thuringiensis* isolates (KAU-11, KAU-51, KAU-166) and the reference strain, HD-1 were subjected to characterization studies.

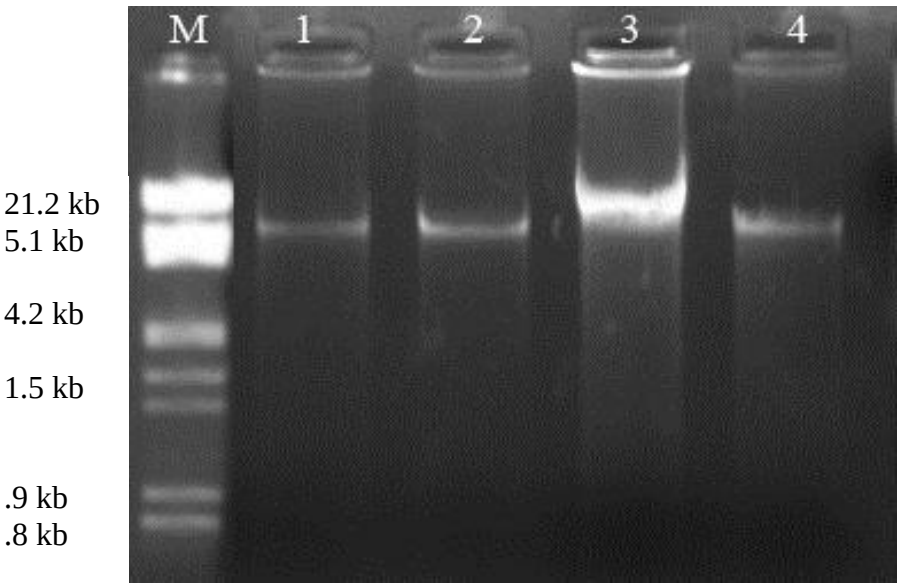
4.3.1 Isolation of total DNA from *B. thuringiensis*

The four *B. thuringiensis* isolates, namely KAU-11, KAU-51, KAU-166 and HD-1 were subjected to DNA isolation. The quality of the isolated DNA was assessed using agarose gel electrophoresis. The bands of DNA were intact (Plate- 7).

4.3.2 Estimation of quantity and quality of DNA

The quantity and quality of DNA were assessed by spectrophotometry and was given in Table 9. The ratio, $OD_{260/280}$ ranged from 1.69 to 2.13.

Plate - 7 Agarose gel electrophoresis of the isolated DNA



M – Molecular weight marker λ DNA/ *Eco* R1+ *Hind* III
Lanes 1- KAU-11, 2- KAU-51, 3- KAU-166, 4- HD-1

Table 9. Quantity and quality of DNA

Isolates	**Quantity (ng/ µl)**	**Absorbance (OD_{260}/OD_{280})**
KAU-11	3061.5	2.13
KAU-51	2173.7	2.13
KAU-166	2277.6	1.98
HD-1	366.9	1.69

4.3.3 Screening of random primers for RAPD

Twenty randomly selected decamer primers of different series were used for screening. The genomic DNA of the isolate KAU-166 was used for screening. On screening, amplifications were observed in 16 primers. The number of bands per primer ranged from one to seven. The numbers of bands produced by different primers are given in Table 10.

Table 10. Total amplicons observed in screening of primers with *Bacillus thuringiensis* isolate, KAU-166.

Sl. No.	Primer	No. of bands/ primer
1	OPF 3	6
2	OPF5	6
3	OPF 11	1
4	N 6	6
5	S 01	6
6	S03	1
7	S05	7
8	S 07	6
9	S 12	1
10	S 22	6
11	S 23	6
12	S 28	2
13	S 30	6
14	S 31	1
15	S 63	2
16	S 103	6

During screening, 10 primers were found to produce more than five bands. These 10 primers were used for RAPD-PCR analysis. The list of selected primers is given in Table 11.

Table 11. Selected decamer primers for RAPD analysis

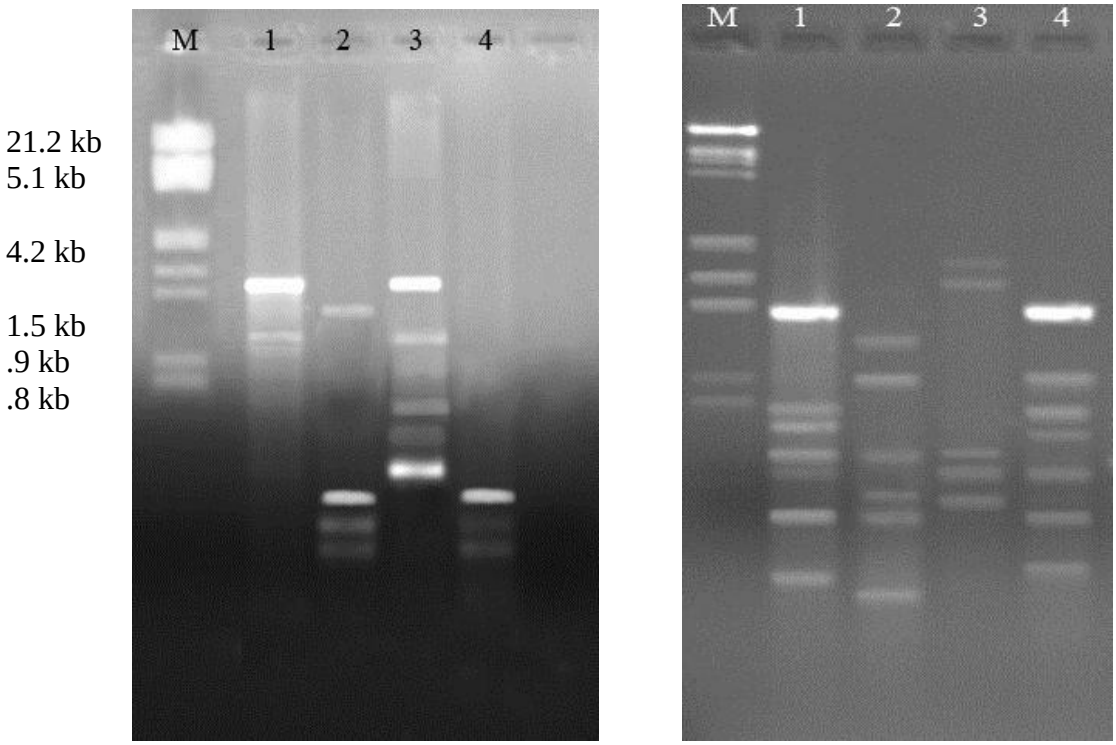
Sl. No.	Primers
1	OPF 3
2	OPF 5
3	N 6
4	S 01
5	S 05
6	S 07
7	S 22
8	S 23
9	S 30
10	S 103

4.3.4 RAPD-PCR

Molecular characterization of the lepidopteran specific isolates of *B. thuringiensis, viz*., KAU-11, KAU-51, KAU-166 and the reference strain, HD-1 was carried out with the selected 10 primers out of twenty primers screened. The amplification patterns produced by the primers are presented in Plate 8 to Plate12.

Table 12 illustrates the number of amplicons obtained for three *B. thuringiensis* isolates and the reference strain with each of ten selected primers

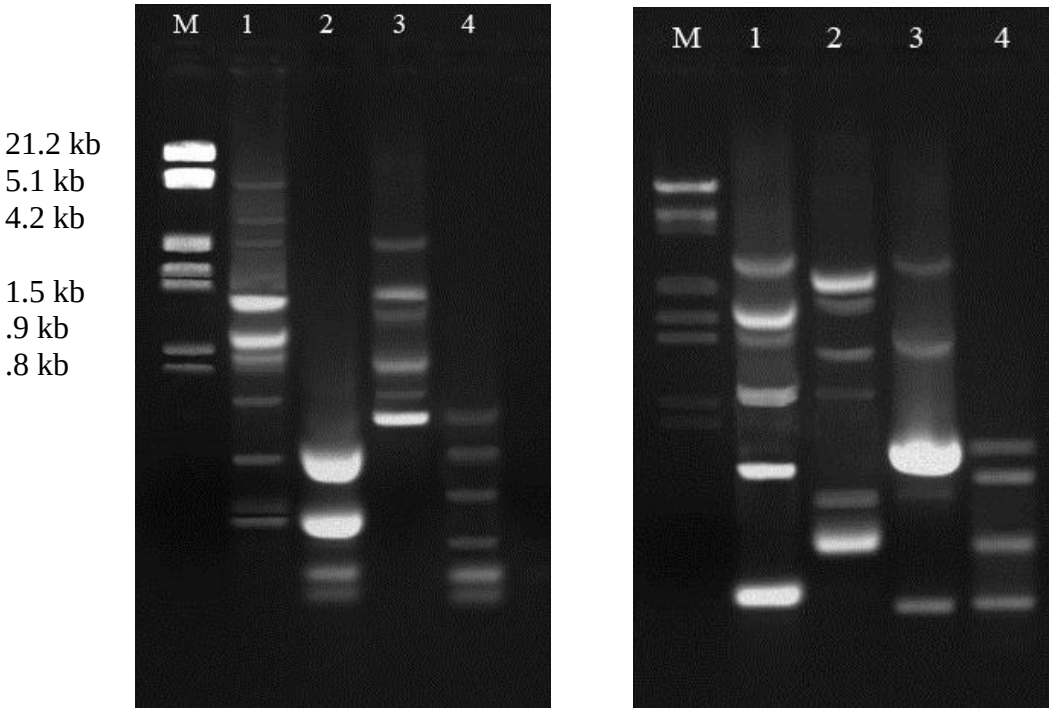
Plate - 8 RAPD profiles of *Bacillus thuringiensis* isolates and standard strain, HD-1 with OPF 3 and OPF 5 primers



a) OPF 3 **b) OPF 5**

M – Molecular weight marker λ DNA/ *Eco* R1+ *Hind* III
Lanes 1- KAU-11, 2- KAU-51, 3- KAU-166, 4- HD-1

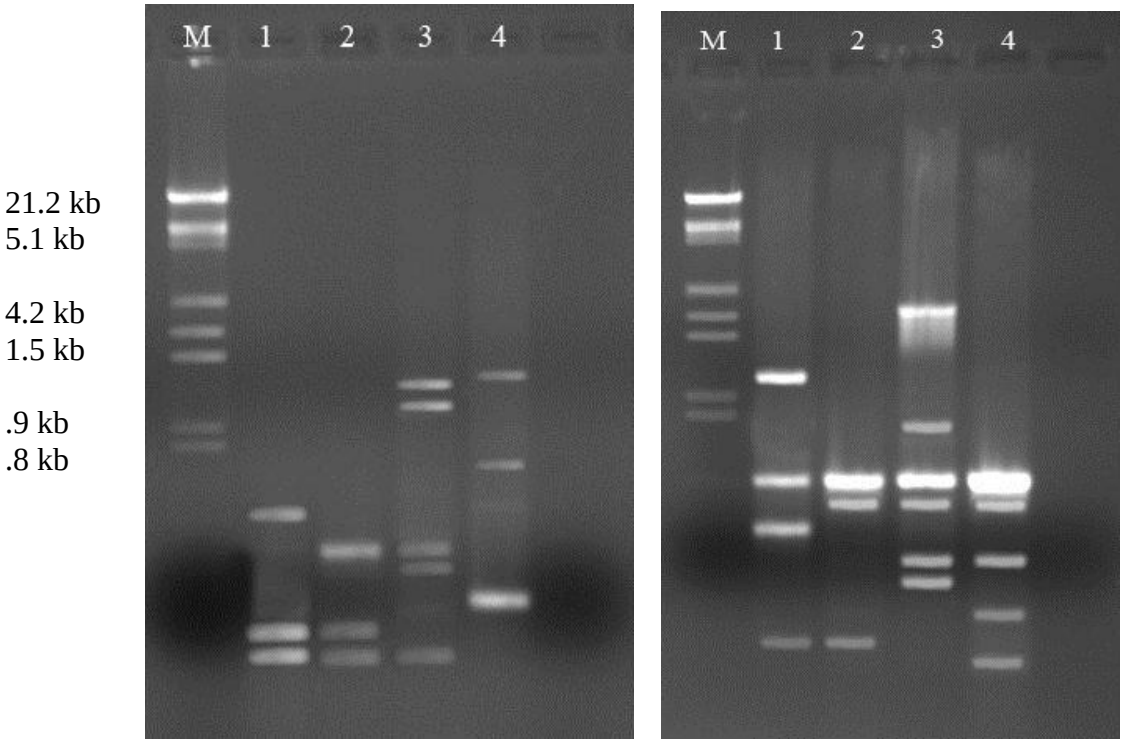
Plate -9 RAPD profiles of *Bacillus thuringiensis* isolates and standard strain, HD-1 with N 6 and S01 primers



c) N 6 **d) S 01**

M – Molecular weight marker λ DNA/ *Eco* R1+ *Hind* III
Lanes 1- KAU-11, 2- KAU-51, 3- KAU-166, 4- HD-1

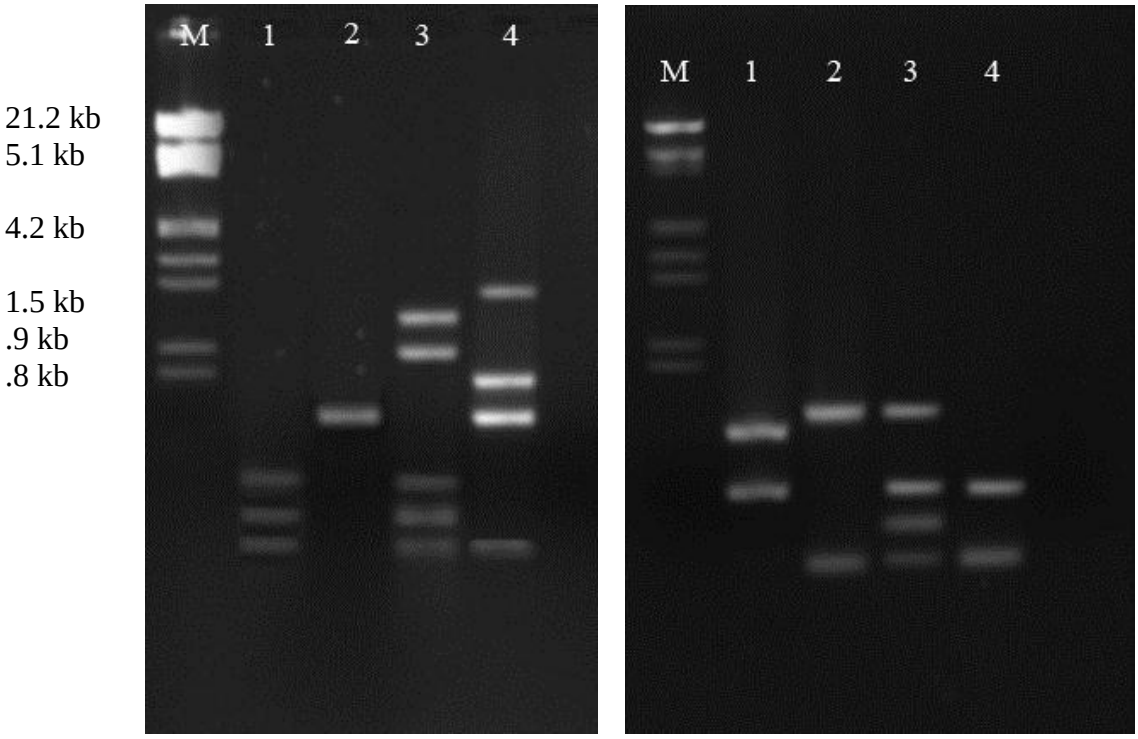
Plate - 10 RAPD profiles of *B. thuringiensis* isolates and standard strain, HD-1 with S 05 and S 07 primers



e) S 05 **f) S 07**

M – Molecular weight marker λ DNA/ *Eco* R1+ *Hind* III
Lanes 1- KAU-11, 2- KAU-51, 3- KAU-166, 4- HD-1

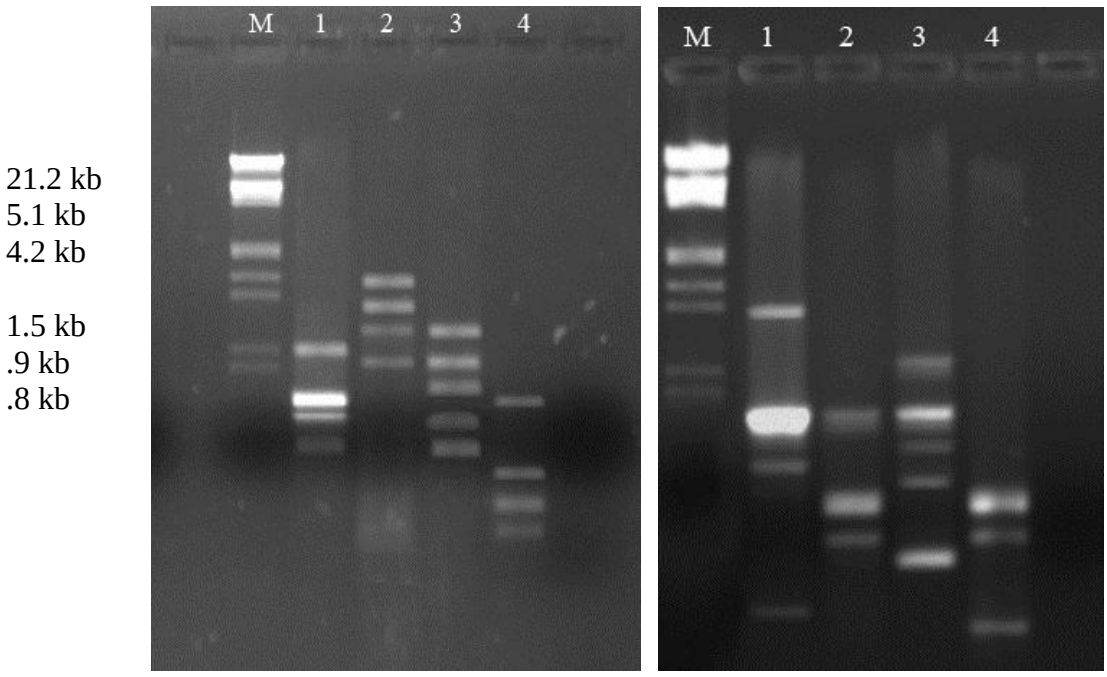
Plate - 11 RAPD profiles of *Bacillus thuringiensis* isolates and standard strain, HD-1 with S 22 and S 23 primers



g) S 22 **h) S 23**

M – Molecular weight marker λ DNA/ *Eco* R1+ *Hind* III
Lanes 1- KAU-11, 2- KAU-51, 3- KAU-166, 4- HD-1

Plate - 12 RAPD profiles of *B. thuringiensis* isolates and standard strain, HD-1 with S 30 and S 103 primers



i) S 30 **j) S 103**

M – Molecular weight marker λ DNA/ *Eco* R1+ *Hind* III
Lanes 1- KAU-11, 2- KAU-51, 3- KAU-166, 4- HD-1

Table 12. Amplification pattern of *Bacillus thuringiensis* isolates with ten selected random primers.

Sl. No.	Primer	Isolate			
		KAU-11	**KAU-51**	**KAU-166**	**HD-1**
1	OPF 3	2	4	5	3
2	OPF 5	7	6	5	7
3	N 6	10	4	6	6
4	S 01	6	6	5	4
5	S 05	3	3	5	3
6	S 07	4	3	6	5
7	S 22	3	1	5	4
8	S 23	2	2	4	2
9	S 30	4	4	5	4
10	S 103	4	3	5	3

The total number of amplicons, number of polymorphic bands, number of monomorphic bands and per cent polymorphism obtained in RAPD with 10 selected random primers were worked out for each primer and were given in Table 13.

All the primers showed high differentiating power, which indicated the ability of primers to expose the genetic diversity of the selected isolates. Table 14 gives information about the differentiating power of different primers.

Table 13. Details of RAPD of selected isolates and HD-1

Sl. No.	Primers used	Total number of amplicons	Number of polymorphic bands	Number of monomorphic bands	Per cent polymorphism
1	OPF 3	9	9	0	100.0
2	OPF 5	14	14	0	100.0
3	N 6	15	15	0	100.0
4	S 01	12	12	0	100.0
5	S 05	8	8	0	100.0
6	S 07	11	10	1	90.9
7	S 22	8	8	0	100.0
8	S 23	5	5	0	100.0
9	S 30	13	13	0	100.0
10	S 103	11	11	0	100.0

Table 14. Differentiating powers of primers used in RAPD analysis of *Bacillus thuringiensis* isolates

Sl. No.	Primer	Total number of phenotypes	Total number of RAPD phenotypes	Differentiating power (%)
1	OPF 3	4	4	100.00
2	OPF 5	4	4	100.00
3	N 6	4	4	100.00
4	S 01	4	4	100.00
5	S 05	4	4	100.00
6	S 07	4	4	100.00
7	S 22	4	4	100.00
8	S 23	4	4	100.00
9	S 30	4	4	100.00
10	S 103	4	4	100.00

4.3.4.1 OPF 3

The primer, OPF 3 was selected for the characterization of four *B. thuringiensis* isolates. Nine amplicons were produced by this primer. No monomorphic bands were obtained giving 100 per cent polymorphism.

4.3.4.2 OPF 5

A total of 14 amplicons were obtained after DNA amplification. All the 14 amplicons were polymorphic giving 100 per cent polymorphism.

4.3.4.3 N 6

The primer, N 6 gave 15 amplicons. It gave 100 per cent polymorphism as all the bands produced were polymorphic. This primer produced the maximum amplification (15) among the 10 selected primers.

4.3.4.4 S 01

Hundred per cent polymorphism was obtained for all the isolates with S 01 primer. It produced a total of 12 amplicons after DNA amplification. No monomorphic bands were observed.

4.3.4.5 S 05

The primer, S 05 produced eight amplicons after PCR amplification. All the eight bands were polymorphic giving 100 per cent polymorphism.

4.3.4.6 S 07

A total of 11 amplicons were produced by the primer, S 07. Per cent polymorphism was 90.9 per cent with a total of 10 polymorphic bands. This was the only primer which produced a monomorphic band after DNA amplification.

4.3.4.7 S 22

Eight amplicons were generated by the primer S 22 after PCR amplification. All the bands produced were polymorphic and no monomorphic bands were observed.

4.3.4.8 S 23

RAPD profile generated by the primer S 23 displayed a total of five amplicons. It produced 100 per cent polymorphism.

4.3.4.9 S 30

The primer, S 30 generated 13 amplicons in RAPD profile. The numbers of polymorphic bands were 13, giving 100 per cent polymorphism.

4.3.4.10 S 103

A total of 11 amplicons were obtained after DNA amplification with the primer, S 103. All the bands produced were polymorphic.

4.3.4 Genetic analysis

The RAPD data was used to generate a similarity matrix using the NTSyS programme.

The phenetic representation of similarity co-efficients among four *B. thuringiensis* isolates were presented in Fig. 2. In the dendrogram, all the four *B. thuringiensis* isolates were divided into two distinct major clusters, '1' and '2' at 27 per cent similarity. The first major cluster comprised of two isolates KAU-11 and KAU-166, which showed maximum similarity between the isolates. The second major cluster formed a sub cluster in which KAU-51 and HD-1 were grouped together. This showed the least similarity of these isolates with the other two isolates, KAU-11 and KAU-166.

Fig. 2 Dendrogram showing the similarity of *B. thuringiensis* isolates

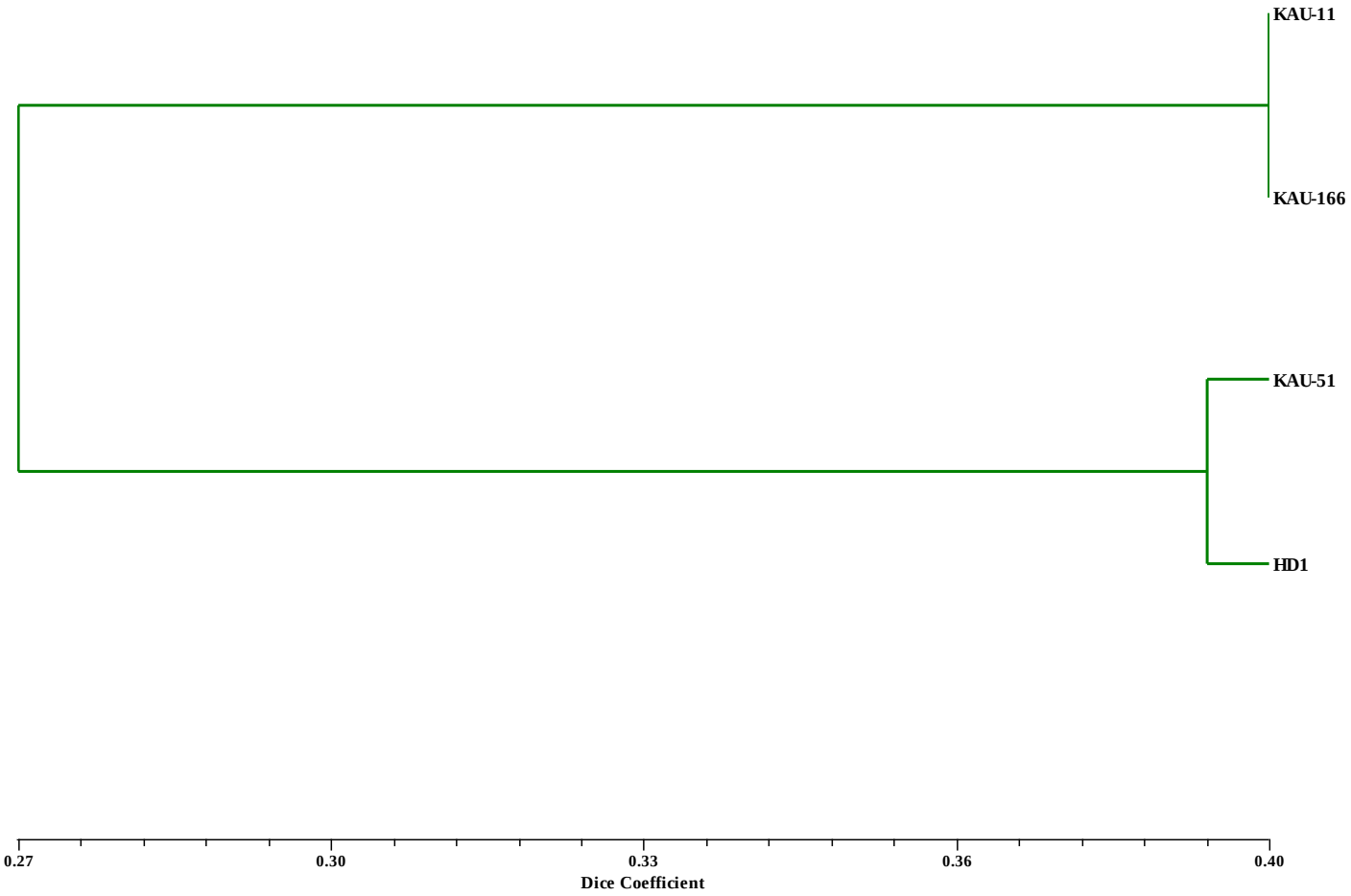


Table 15. Similarity coefficient of *Bacillus thuringiensis* isolates

	KAU-11	**KAU-51**	**KAU-166**	**HD-1**
KAU-11	1.0000000			
KAU-51	0.2469136	1.0000000		
KAU-166	0.3958333	0.2758621	1.0000000	
HD-1	0.3023256	0.3896104	0.2608696	1.0000000

The similarity coefficients between *B. thuringiensis* isolates ranged from 0.24 to 0.39. The maximum similarity was seen between the isolates, KAU-11 which was collected from Thusharagiri (Calicut Dt.) and KAU-166 which was collected from Nelliyampathi (Palakkad Dt.) with a similarity coefficient of 0.39 and minimum similarity was observed between KAU-11 and KAU-51 with a coefficient of 0.24.

Discussion

5. DISCUSSION

The present study was conducted with 20 native *Bacillus thuringiensis* isolates collected from the Western Ghat Area of Kerala for their lepidopteran specificity and their molecular characterization by RAPD-PCR technique. The results of the study were discussed herewith.

5.1 Preliminary screening of native isolates of *B. thuringiensis* against *Spodoptera litura*

In the present study, the tobacco caterpillar, *S. litura* was used as the test insect to find the bioactivity of the soil isolated bacterial isolates. Western Ghats from which the bacteria were collected is considered as the hot spot of biodiversity. The normal habitat of *B. thuringiensis* was reported to be soil (Dulmage and Aizawa, 1982). The study conducted by deLucca *et al*. (1981) also showed that *B. thuringiensis* could be found in soil samples.

During the preliminary screening of 20 isolates, three native isolates, namely, KAU-11, KAU-51, and KAU-166 were observed to give high mortality while two isolates namely, KAU-45 and KAU-130 caused three per cent mortality.

The number of lepidopteran effective *B. thuringiensis* isolates among those isolated from soil were reported to be always limited (Asalmol *et al*., 2004). In the present study, out of the 20 soil isolated *B. thuringiensis*, only three were found to be lepidopteran specific. This could be corroborated with the work of Hernandez (1988) who tested 52 *B. thuringiensis* isolates against *S. frugiperda*. He found that only two isolates caused high mortality (100 %) to the larvae. Bohorova *et al*. (1996) tested 352 native isolates of *B. thuringiensis* against *S. frugiperda* and only one was observed to cause mortality between 70 to 80 per

cent. Valicente and Barreto (2003) reported only 62 *B. thuringiensis* strains, which caused 81 to 100 per cent mortality to *S. frugiperda* larvae when they tested 3408 strains of *B. thuringiensis*, collected from the soil samples of 10 Brazilian states. The ineffective isolates could be non pathogenic or avirulent to *S. litura.* Ten isolates of *B. thuringiensis* pathogenic to *H. armigera* were reported by Asalmol *et al*. (2004) from the soil of Vidarbha out of a total of 17 isolates. Limited pathogenicity of *B. thuringiensis* products against *S. litura* was reported (Whitlock *et al*., 1991; Federici, 1999).

5.1.1 Symptoms of *B. thuringiensis* infection

Cessation of feeding was the first symptom showed by the larvae of *S. litura* after feeding on the *B. thuringiensis* treated leaf discs. Angus (1956) and Cooksey (1971) reported that the first sign of poisoning following ingestion of crystalline endotoxin was found to be paralysis of gut and mouth parts leading to cessation of feeding. Feeding inhibition has been reported as an immediate effect as a protective action (Angus, 1956). Brunner and Stevens (1986) also reported the cessation of feeding soon after the ingestion of bacteria.

Larvae stopped moving and mortality occurred within 19 to 20 h. after feeding. Larval body became putrified after death and changed to black coloured mass. Oozing of brown fluid from larval body was also observed. Similar observations were reported by Brunner and Stevens (1986) and Wilding (1986). Similar results were also obtained by Neema (2007) on the larvae of pumpkin caterpillar, *Diaphania indica* S.

5.1.2 Mortality of *S. litura* larvae

Preliminary screening of the 20 isolates showed that only three isolates, namely, KAU-11, KAU-51, KAU-166 and the reference strain HD-1 indicated a variation in the mortality caused by them at a specific concentration ($1x10^9$ spores/ml). The result was in agreement with Prabagaran *et al*. (2002) who obtained only one efficient strain of *B. thuringiensis* out of 831 tested against

second instar larvae of *S. litura*. Similar results were observed by Valicente and Barreto (2003). Maghodia and Vyas (2004) found that native isolate, GAU CT2 caused 66.4 per cent mortality to larvae of *A. janata* at $1x10^9$ spores per ml. In the present study, the mortality in all the *B. thuringiensis* isolates and HD-1 ranged from 76.7 per cent to 96.7 per cent. During screening, the reference strain was observed to be more efficient than other isolates. The experiment clearly indicated the differences in the insecticidal activity among the isolates. This result agreed with that of Kaur *et al*. (2006) who reported the difference in insecticidal activity among the four *B. thuringiensis* strains, namely, MTCC 868, Bt 5, Bt 9 and 4D4 against the second instar larvae of *P. xylostella*. The finding is also in agreement with that of Justin *et al*. (1989) who reported the variation in insecticidal activity among various *B. thuringiensis* subspecies.

5.2 Bioassay of the selected isolates of *B. thuringiensis*

Pathogenicity studies carried out with five different concentrations, namely, $1x10^7$,$1x10^8$, $1x10^9$, $1x10^{10}$, $1x10^{11}$ spores per ml showed an increase in the per cent mortality with the increase in the concentration of spore suspension (Fig. 3 to Fig. 6). This result is in conformity with the finding of Sareen *et al*. (1983) who described the increase in the per cent mortality with the increase in the spore concentrations. Variations in the effectiveness among different concentrations of spore or crystal toxins of *B. thuringiensis* were also reported (Ahmed *et al*., 1988; Zaz, 1989).

Spores were observed to have insecticidal activity (Scherrer and Somerville, 1977; Somerville and Pockett, 1975) and have synergism with the small amount of toxin in the spore preparations (Liu *et al*., 1998). They also reported that the synergistic effect of *B. thuringiensis* subsp. *kurstaki* spores against a resistant strain of diamondback moth were equal to or greater than those against a susceptible strain.

Fig. 3 Mortality caused by different concentrations of *Bacillus thuringiensis* isolate, KAU-11

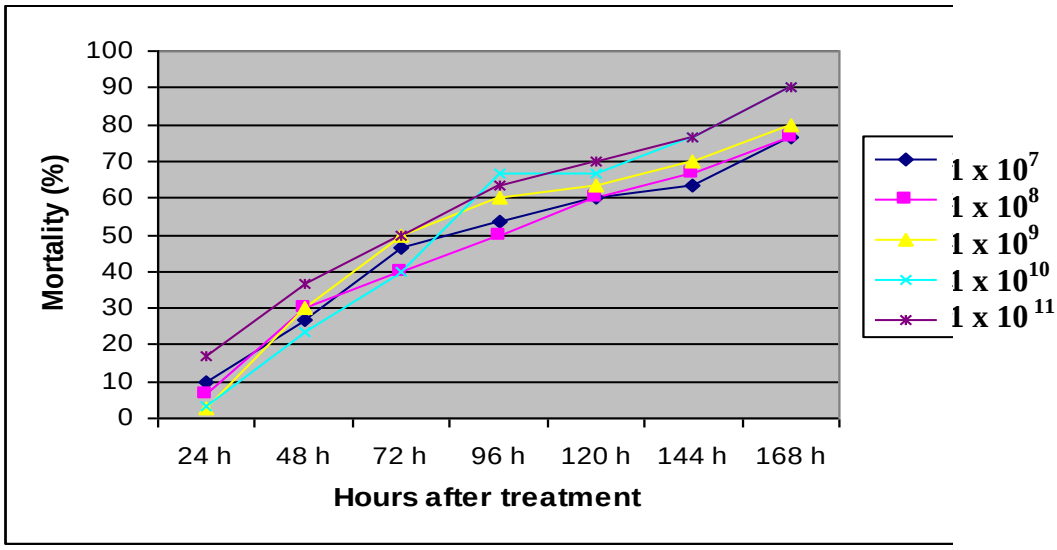


Fig. 4 Mortality caused by different concentrations of *Bacillus thuringiensis* isolate, KAU-51

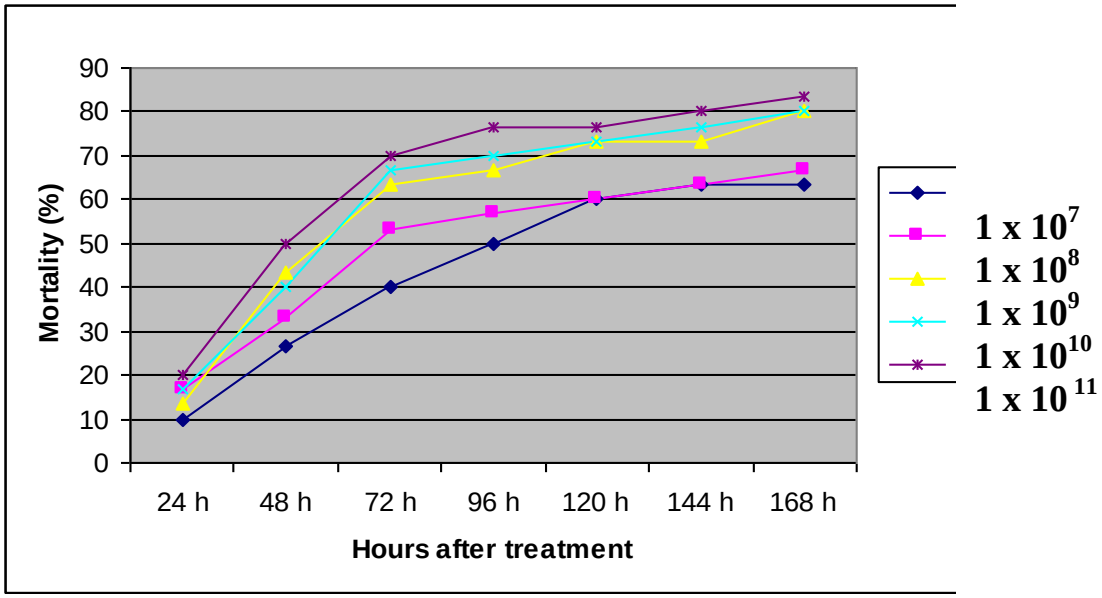


Fig. 5 Mortality caused by different concentrations of *Bacillus thuringiensis* isolate, KAU-166

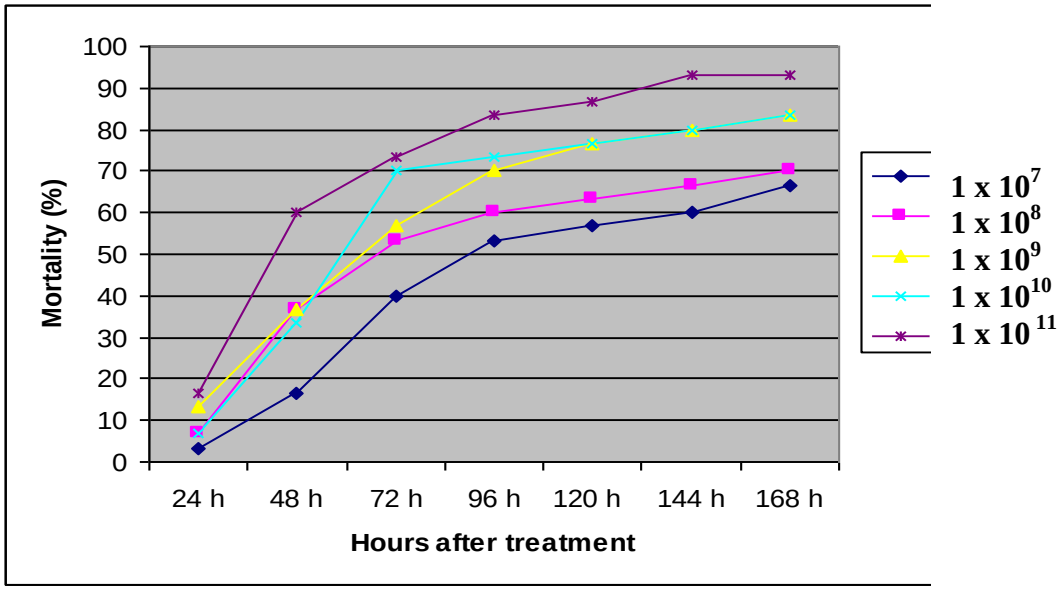
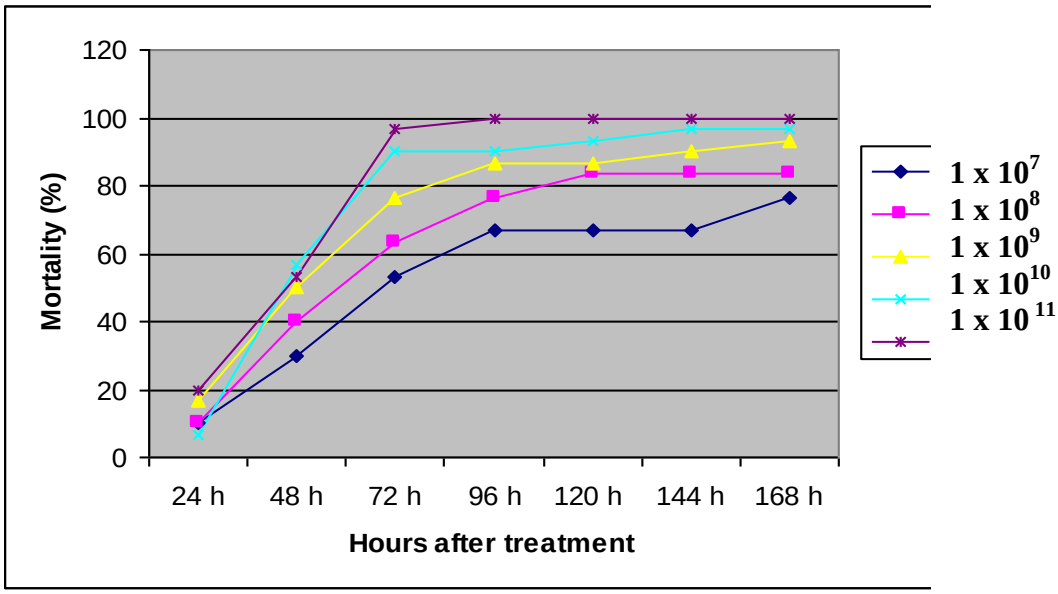


Fig. 6 Mortality caused by different concentrations of HD-1



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Isolates, which were selected as lepidopteran specific, were found to have bipyramidal crystals (Plate- 13). This could be correlated with the study conducted by Travers *et al*. (1987) who showed that *B. thuringiensis* isolates that produced bipyramidal crystals were lepidopteran active as compared to the *B. thuringiensis* isolates that produced other types of crystals *viz*., spherical, oval, rhomboidal and irregular. The study conducted by Asokan and Puttaswamy (2007), also showed that the isolates that produced bipyramidal crystals only were toxic to *P. xylostella*. Scanning Electron Microscopy (SEM) analysis conducted in the study of Gitahy *et al*. (2007) revealed large amount of bipyramidal crystals similar to those found in other *B. thuringiensis* strains with entomopathogenic activity against Lepidoptera. Analysis of the cumulative per cent mortality showed that different concentrations of the isolates KAU-11, KAU-51 and KAU-166 were on par in the bio-efficacy against *S. litura*. This shows the possibility of utilizing these selected isolates in the management of this polyphagous pest, *S. litura*.

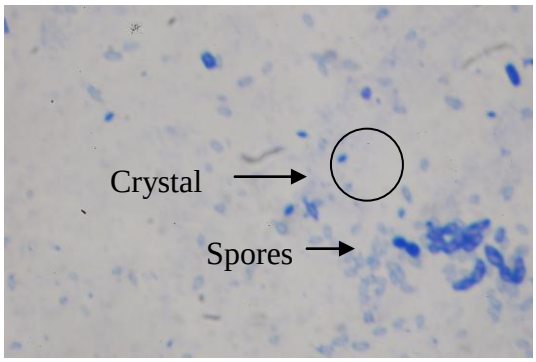
5.2.1 Median Lethal Concentration (LC_{50})

The dose mortality response of *S. litura* to the different concentrations of *B. thuringiensis* isolates indicated a slight variability among the isolates to kill the insects (Fig. 7). The lowest LC_{50} was observed for KAU-51 which indicated the comparatively high effectiveness of the isolate. Such differences in the insecticidal activity of different isolates of *B. thuringiensis* might be ascribed to the difference in the carbohydrate affinity of the domain II of the cry proteins resulting in variable binding specificity with the receptors at the brush border membrane of the insect larvae leading to differences in toxicity of the cry protein (Smedley and Ellar, 1996).

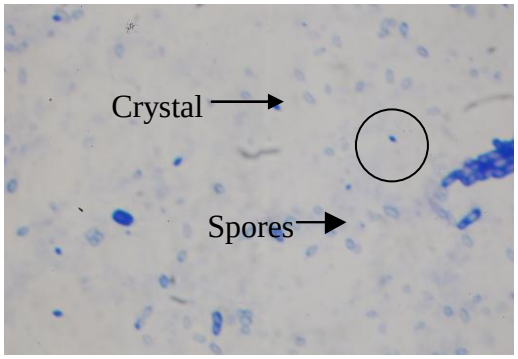
5.2.2 Median Lethal Time (LT_{50})

The time mortality response of *B. thuringiensis* against *S. litura* revealed that the standard strain, HD-1 had the faster knock down action. The time (hours) to cause 50 per cent mortality was recorded as 68.0 h., 57.5 h., 48.0 h., 44.4 h. and 45.4 h. for the concentrations $1x10^7$, $1x10^8$**,** $1x10^9$**,** $1x10^{10}$ and $1x10^{11}$ (spores/ml)

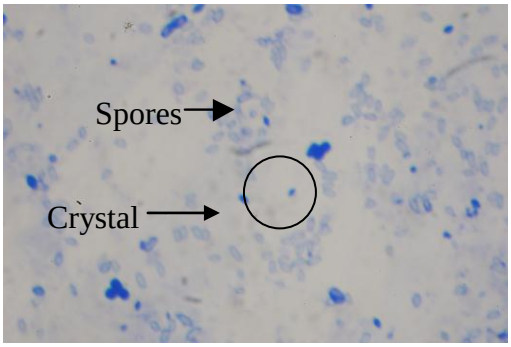
Plate - 13 Crystal proteins produced by *Bacillus thuringiensis* isolates and the reference strain, HD-1



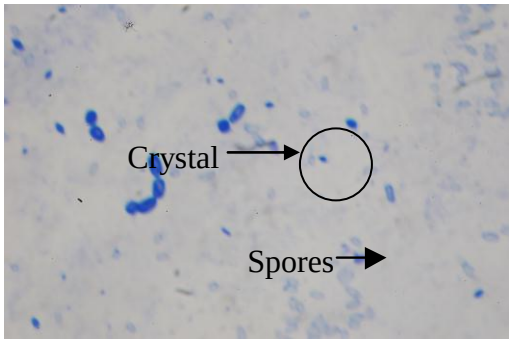
a) KAU-11



b) KAU-51

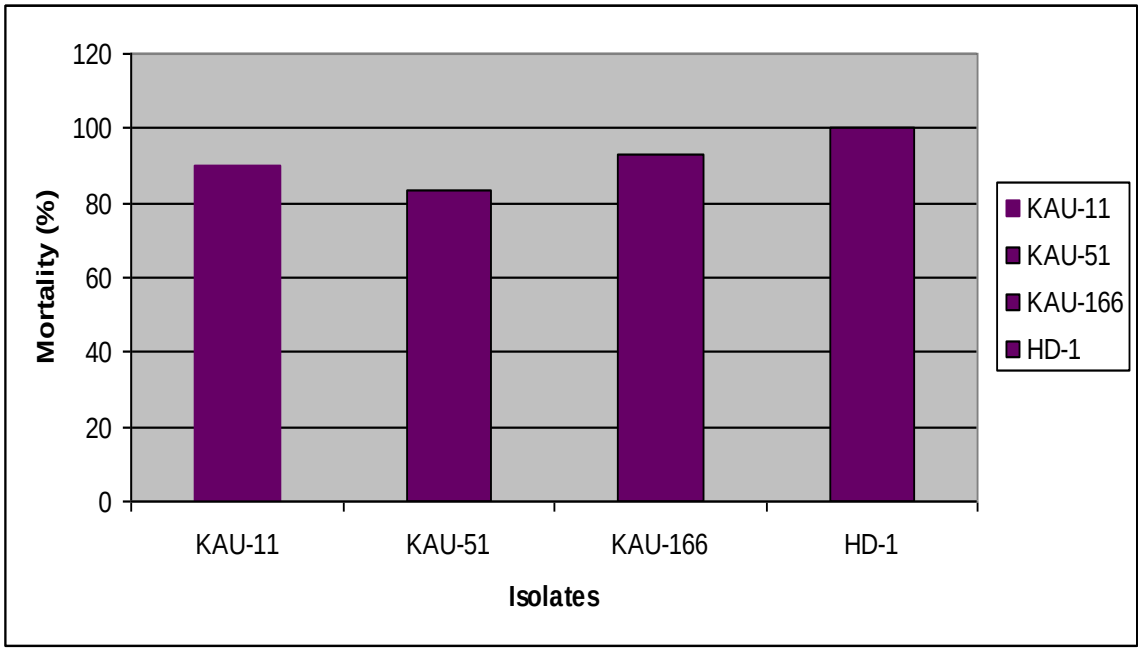


c) KAU-166



d) HD-1

Fig. 7 Mortality of *Spodoptera litura* at the highest concentration



respectively. This showed the efficacy of the strain to cause mortality within a short time. The synergistic effect of spores was facilitated by the activity of specific crystal proteins and resulted in the quick killing action of the isolate. It was reported that the spores contaminated the haemocoel through a channel created in the midgut membrane and multiplied at a faster rate utilizing the nutrients from haemolymph (Ali *et al*., 1985; Wilson and Benoit, 1990; Borgonie *et al*., 1995) which might lead to bacterial septicemia and death of larvae within two to three days of ingestion (Ali *et al*., 1985).

5.3 Molecular characterization of *B. thuringiensis* isolates

5.3.1 DNA isolation

Isolation of good quality DNA is a pre-requisite for RAPD assay. Different methods are given by different workers for the isolation of genomic DNA of *B. thuringiensis*. Different workers used different methods for the extraction of genomic DNA of *B. thuringiensis* using Wizard ® Genomic DNA Putrification Kit (Promega, USA, part number A1120) (Sadder *et al*., 2006), D Neasy Minikit (Gene Company, Limited, Guangzhou, China (Salem *et al*., 2006).

In the present study, the extraction of genomic DNA of four *B. thuringiensis* isolates was done following the protocol of Sambrook and Russel (2001). The same procedure was used by Rajesh *et al*. (2006) for the DNA extraction of *Bacillus thuringiensis* isolates. Isolated DNA was analysed by agarose gel electrophoresis using 0.8 per cent agarose gel. The bands of DNA were intact. This indicated that the quality of DNA was good.

The ratio of absorbance at 260 nm and absorbance at 280 nm ($OD_{260/280}$) ranged from 1.63 to 2.13. The value of $OD_{260/280}$ between 1.8 to 2.0 indicates relatively pure DNA. An increase in the value may be due to the presence of contaminating molecules. Ratios below 1.8 may be due to the presence of proteins.

5.3.2 RAPD analysis

Different molecular markers are being used for molecular characterizations. The PCR based markers for the characterization studies include random amplified polymorhic DNA (RAPD), amplified fragment length polymorphism (AFLP), microsatellites or simple sequence repeat polymorphism (SSRP), arbitrary primered PCR (AP-PCR) etc. AFLP is a high sensitive method for detecting polymorphism through out the genome. It is essentially a combination of RFLP and RAPD methods. AP-PCR is a special case of RAPD wherein discreet amplification patterns are generated by employing single primers of 10-50 bases in length in PCR of genomic DNA.

In the present study RAPD-PCR was selected as the molecular marker as it was fast and informative in differentiating the *B. thuringiensis* strains. For RAPD-PCR, the information of any sequence was not needed in advance. It has proved to be an informative method suitable for the study of a large number of strains with in a short time.

The numerous imponderables of RAPD-PCR make it impossible to gain reliable results with this method. Besides the lack of reproducibility associated with RAPD-PCR results, another disadvantage of this method is the lack of universally established guidelines for the selection of primers.

Random Amplified Polymorphic DNA assay detects nucleotide sequence polymorphism in a DNA amplification based assay using only a single arbitrary nucleotide sequence (Tingey and delTufo, 1990). In RAPD, arbitrary primers were used. It recognizes differences in prevalence and positions of annealing sites in the genome producing sets of fragments that are considered to reflect the genomic composition of the strain (Rivera and Priest, 2003).

Twenty randomly selected decamer primers were used in the study. These random primers were obtained from Operon technologies, USA and IDT, USA. Number of bands during screening ranged from one to six. Out of the twenty primers, ten primers, which produced more than five bands, were selected for RAPD analysis. A negative control was also run for each reaction to ensure that the amplification produced was originated from genomic DNA and not from primer artifacts. No amplification was observed in control reactions indicating that the components of the reaction mixture were free of any contaminants. Total number of amplicons ranged from five (S 23) to fifteen (N6). Hundred per cent polymorphism was observed with all the primers except with the primer, S 07 where it was 90.9 per cent.

The high differentiating power (100%) of the primers showed their ability to reveal the genetic diversity of the *B. thuringiensis* isolates. As RAPD profiles produced by different primers were dissimilar, these primers could be used for conducting RAPD-PCR technique of *B. thuringiensis* isolates.

The RAPD bands in the profiles of ten selected primers were scored as one (to indicate the presence of bands) and zero (to indicate the absence of bands). The pair wise similarity matrix indicated the genomic similarity between the four *B. thuringiensis* isolates. Genetic similarity values computed for all the isolates ranged from 0.24 to 0.39.

The dendrogram developed through UPGMA cluster analysis gave a clear idea of the relatedness among the isolates. The isolates, KAU-11 and KAU-166 showed the highest similarity (0.39). These two isolates were collected from two different locations such as Tusharagiri (Calicut Dt.) and Nelliyampathi (Palakkad Dt.). Similarity between the clones of genomically similar or highly related strains of *B. thuringiensis* from geographically distinct locations was reported as due to the limited exchange of chromosomal DNA (Rivera and Priest, 2003).

The isolate, KAU-51 and the reference strain, HD-1 were grouped together in the sub cluster of second major cluster with a similarity coefficient of 0.38. Clusters produced could give an indication on the toxicity of isolates belonging to clusters in terms of insecticidal similarities with in the same cluster (Sadder *et al*., 2006). The dendrogram indicated high variability among the isolates, selected from the bioassay studies due to the high mortality shown for the test insect. The genetic dissimilarity existing between these isolates will help to prevent the development of resistance to *B. thuringiensis*.

Even though RAPD-PCR was used to study the genetic diversity between the isolates, it was not considered as reproducible until the reaction was carried out in same conditions. It was not advisable to use RAPD analysis alone for the characterization of strains. It could be performed with other biochemical tests to differentiate the isolates.

It can be concluded that RAPD-PCR is a powerful tool to study genomic similarity and/or polymorphisms among bacterial strains from different subspecies. It gives a good opportunity to detect the biodiversity of a group of isolates. The molecular characterization of the bacterial isolates with more number of primers will help to understand the genetic diversity in detail.

Summary

6. SUMMARY

Investigations were undertaken at the Department of Agricultural Entomology and CPBMB, College of Horticulture, Vellanikkara, during 2006 - 2008 to study the "Bio-efficacy and molecular characterization of the native isolates of *Bacillus thuringiensis* Berliner". The salient findings of the present investigation are summarized below.

- Twenty native isolates of *B. thuringiensis* were subjected to preliminary screening to find out the lepidopteran specificity of the isolates against tobacco caterpillar, *S. litura*. Three isolates namely KAU-11, KAU-51, KAU-166 and the reference strain, HD-1 caused 76.7 per cent, 80.0 per cent, 86.7 per cent and 96.7 per cent mortality respectively showing their specificity to the insect.
- Pathogenicity of the three selected lepidopteran specific isolates at different concentrations was tested against *S. litura* by standardized bioassay.
- There was no significant difference in the cumulative mortalities of the three isolates and the reference strain to *S. litura* and this indicated their equal effectiveness with HD-1.
- The LC_{50} values of the isolates were found to be 1.2589×10^6, 6.3095×10^4, 7.9432×10^4 and 1.5849×10^5 spores per ml for KAU-11, KAU-51, KAU-166 and HD-1 respectively.
- The LT_{50} values calculated for all concentrations of the isolates ranged from 44.4 h. to 96.0 h.
- The genomic DNA of the selected isolates was extracted using the protocol of Sambrook and Russel (2001).
- In the DNA quality assessment, DNA bands were found to be intact.
- The $OD_{260/280}$ values of DNA from the selected isolates ranged from 1.69 to 2.13.

- Twenty randomly selected decamer primers were screened for RAPD assay. Sixteen primers produced amplifications during screening.
- RAPD-PCR was carried out for all isolates with 10 primers which produced more than five bands.
- Maximum number of amplicons (15) was produced by primer, N 6 and the primer, S 23 produced the minimum (5).
- Out of the 10 primers, nine primers produced 100 per cent polymorphism while the primer, S 07 produced 90.9 per cent.
- Scoring of RAPD amplification patterns and the analysis of data was done using NTSyS pc (ver 2.0) software to determine the genetic similarity between the isolates.
- Dendrogram was prepared based on the similarity coefficients using UPGMA. The isolates, KAU-11 collected from Thusharagiri (Calicut Dt.) and KAU-166 collected from Nelliyampathi (Palakkad Dt.) showed the maximum similarity coefficient of 0.39.
- HD-1 and KAU-51, which were grouped together in the same sub cluster of second major cluster, showed a similarity coefficient of 0.38.
- A minimum similarity was observed between KAU-51 and KAU-11 with a similarity coefficient of 0.24.

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Annexure

Annexure –I

1. Composition of semi synthetic diet for *Spodoptera litura*

Chickpea flour	-	105.00 g
Sorbic acid	-	1.00 g
Streptomycin sulphate	-	0.25 g
Yeast extract	-	10.00 g
Agar	-	12.75 g
Vitamin –E tablets	-	2 No.s
Multivitamin tablet	-	2 No.s
Ascorbic acid	-	3.25 g
Formalin 10%	-	5.00 ml
Distilled water	-	780.00 ml

2. Composition of different media used in the study

1. Luria Bertani (LB) broth

Tryptone	-	10.0 g
Yeast Extract	-	5.0 g
NaCl	-	5.0 g
Distilled water	-	1l
pH adjusted to	-	7 ± 0.2

2. Luria Bertani Agar medium (LBA medium)

Tryptone	-	10.0 g
Yeast Extract	-	5.0 g
NaCl	-	5.0 g
Agar	-	20.0 g
Distilled water	-	1l
pH adjusted to	-	7 ± 0.2

3 T3 broth

Tryptone	-	3.0 g
Tryptose	-	2.0 g
Yeast Extract	-	1.5 g
Sodium phosphate	-	1.2 g
Distilled water	-	1l
pH adjusted to	-	6.8

4 T3 agar medium

Tryptone	-	3.0 g
Tryptose	-	2.0 g
Yeast Extract	-	1.5 g
Sodium phosphate	-	1.2 g
Agar	-	18.0 g
Distilled water	-	1l
pH adjusted to	-	6.8

Annexure II

Reagents for DNA isolation

1. 10 mM Tris Cl

1 M Tris Cl (pH- 8.0)	-	1.0 ml
Distilled water	-	100.0 ml

2 1000 mM NaCl

1 M NaCl	-	10.0 ml
Distilled water	-	100.0 ml

3 TE buffer

(Tris Cl- 50 mM; EDTA	-	20 mM)
Tris Cl -.05 M (pH-8.0)	-	0.394 g
.02 M EDTA (pH-8.0)	-	0.372 g
Distilled water	-	100.0 ml

4 Lyzozyme stock

Lyzozyme	-	50.0 mg
Distilled water	-	1.0 ml

Stock was prepared by dissolving 50 mg lyzozyme in 1 ml water and was stored under refrigerated conditions.

5 RNase stock

RNase	-	10.0 mg
Distilled water	-	1 ml

Stock was prepared by dissolving 10 mg RNase in 1 ml water and was stored under refrigerated conditions at $^{-}20^{o}$ C.

6 2% SDS in TE buffer

SDS	-	2.0 g
TE buffer	-	100.0 ml

7 Proteinase K

Proteinase K	-	20.0 mg
Distilled water	-	1 ml

Stock was prepared by dissolving 20 mg Proteinase K in 1 ml water and was stored under refrigerated conditions at $^{-}20^{o}$ C.

8 Phenol: Chloroform (1:1 v/v)

To 1 part of Tris saturated phenol, 1 part of chloroform was added.

9 Chloroform : isoamyl alcohol (24:1 v/v)

To 24 parts of chloroform, 1 part of isoamyl alcohol was added and mixed properly. The mixture was stored in refrigerator before use.

10 3M Sodium acetate

Sodium acetate	-	20.412 g
Distilled water	-	50 ml

11 Chilled isopropanol

12 70 % ethylalcohol

To 70 parts of absolute ethanol, 30 parts of double distilled water was added.

Annexure III

Buffers and dyes used in gel electrophoresis

1. 6 x Loading/ tracking dye

Bromophenol blue - 0.25 %

Xylene cyanol - 0.25 %

Glycerol - 30.0 %

The dye was prepared and kept in refrigerator at 4^{o} C.

2 Ethidium bromide

The dye was prepared as a stock solution of 10 mg per ml in water and was stored at room temperature in dark bottle.

3 50X TAE buffer

Tris base - 242.0 g

Glacial acetic acid - 57.1 ml

0.5 M EDTA (pH 8.0) - 100.0 ml

Distilled water - 1000.0 ml

The solution was prepared and stored at room temperature.

Abstract

Bio-efficacy and molecular characterization of the native isolates of *Bacillus thuringiensis* Berliner

By

Jyothi Sara Jacob

ABSTRACT OF THE THESIS

Submitted in partial fulfilment of the requirement for the degree of

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ABSTRACT

Bacillus thuringiensis Berliner shortly known as *B.t.*, is a gram-positive, spore forming bacterium that produces proteinaceous crystal containing insecticidal toxins. The host range of *B.t.* has expanded considerably due to extensive screening programmes. By virtue of its lack of toxicity towards other species of animals and human beings, *B.t.* has emerged as a safe candidate in the IPM programmes of various agroecosystems.

Frequent exposure to one type of *B. thuringiensis* toxins can develop resistance in insects. Isolation of novel strains is important to overcome the onset of insect resistance.

The present study on "Bio-efficacy and molecular characterization of native isolates of *Bacillus thuringiensis* Berliner" was conducted at the Department of Agricultural Entomology and CPBMB, College of Horticulture, Vellanikkara during the period from 2006 to 2008 with an objective to study the pathogenicity of 20 *B. thuringiensis* isolates collected from the Western Ghats of Kerala, one of the well known hot spots of biodiversity. The tobacco caterpillar, *Spodoptera litura* Fb. which was used as the test insect was reared in semi-synthetic diet (Ballal, 2004) and also in natural diet on castor leaves.

The preliminary screening was performed for lepidopteran specificity with the ideal dose of 1×10^9 spores per ml showed that three isolates were toxic to *S. litura*. The per cent mortality caused by the lepidopteran effective isolates, namely, KAU-11, KAU-51, KAU-166 and the reference strain, HD-1 were 76.7 per cent, 80.0 per cent, 86.7 percent, 96.7 per cent respectively.

A standardized bioassay was carried out with five different concentrations of all the selected isolates namely, KAU-11, collected from Thusharagiri (Calicut Dt.), KAU-51 from Kolahalamedu (Idukki Dt.), KAU-166 from Nelliyampathi

(Palakkad Dt.) and the reference standard HD-1 (*B. thuringiensis* subsp. *kurstaki*). The mortality ranged from 63.3 to 100.0 per cent in various isolates and there was no significant difference between isolates. This indicated the equal effectiveness of the native isolates with the standard strain, HD-1. The LC_{50} value for the isolates was calculated by Finney's Method of Probit Analysis. The lowest LC_{50} was obtained in KAU-51, with 6.3095×10^4 spores per ml and highest in KAU-11, with 1.2589×10^6 spores per ml. The lethal time to cause 50 per cent mortality (LT_{50}) ranged from 44.4 h. to 96.0 h. in different isolates. The lowest LT_{50} was recorded in HD-1, which shows the ability of the standard to cause mortality slightly earlier compared to other isolates.

The molecular characterization of the selected isolates was performed with RAPD-PCR technique. RAPD-PCR is a simple and rapid method for determining genetic diversity in various organisms and is a means of creating a biochemical finger print of an organism. Out of the 20 primers screened, 10 primers which produced more than five bands were selected for RAPD analysis. The RAPD data was used to generate a similarity matrix using the NTSyS programme. Clustering was done and dendrogram was drawn using Unweighted Pair Group Method of Arithematic Averages (UPGMA). The results showed that high variability exists between the selected isolates.

Further studies are required to identify the subspecies of the efficient *B.t.* isolates to evaluate the field effectiveness against *S. litura* and other major lepidopteran pests for its utilization in pest management programmes. More primers need to be screened to study the genetic diversity of the isolates.