Survey, isolation and characterization of entomopathogenic bacteria of some sporadic lepidopteran pests of tea foliage from Darjeeling foothills and plains

A thesis submitted to the University of North Bengal

for the award

of

Doctor of Philosophy

in

Zoology

By

Sangita Khewa Subba, M.Sc

Supervisor

Prof. Ananada Mukhopadhyay

Co-supervisor

Dr. Min Bahadur

Department of Zoology

University of North Bengal

November, 2017

DECLARATION

I declare that the thesis entitled "Survey, isolation and characterization of entomopathogenic bacteria of some sporadic lepidopteran pests of tea foliage from Darjeeling foothills and plains" has been prepared by me under the guidance of Prof. Ananda Mukhopadhyay and Dr. Min Bahadur, Department of Zoology, University of North Bengal. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

Sangita Khewa (Subba).

Sangita Khewa (Subba)
Entomology Research Unit
Department of Zoology
University of North Bengal
P.O. NBU, Siliguri
District: Darjeeling-734013
West Bengal, India

Date: 20·11·2017

UNIVERSITY OF NORTH BENGAL

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P.O. North Bengal University,
Raja Rammohunpur, Dt. Darjeeling,
West Bengal, India, PIN - 734 013

From

Supervisors,

CERTIFICATE

This is to certify that Ms. Sangita Khewa (Subba) has prepared the thesis entitled '**Survey, isolation and characterization of entomopathogenic bacteria of some sporadic lepidopteran pests of tea foliage from Darjeeling foothills and plains**' for the award of PhD degree of the University of North Bengal, under our guidance and supervision. She has carried out the work at the Department of Zoology, University of North Bengal.

SUPERVISOR

Dr. Ananda Mukhopadhyay
Professor (Retired)
Department of Zoology
University of North Bengal
15./11/17

CO-SUPERVISOR

Dr. Min Bahadur
Associate Professor
Department of Zoology
University of North Bengal
15/11/17

Phone: (0353) 2776353, Fax: (0353) 2699 001, E-mail: zoology_nbu@rediffmail.com, Visit us at: www.nbu.ac.in

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Sangita Khema (Subba) 16/11/17

SANGITA KHEMA (SUBBA)
ENTOMOLOGY RESEARCH UNIT
DEPT. OF ZOOLOGY
UNIVERSITY OF NORTH BENGAL

16/11/17
Dr. M. Bahadur
Associate Professor
Department of Zoology
University of North Bengal
Siliguri- 734013, W.B.

16.11.17
Dr. Ananda Mukhopadhyay
Ex Professor
Department of Zoology
University of North Bengal
P.O.- NBU, Darjeeling- 734013

ABSTRACT

Tea, *Camellia sinensis* (L.) O. Kuntze, has earned the reputation of being a cheap and readily available beverage. This great popularity of tea draws attention of almost all tea producing countries from the economic point of view. Tea is an intensively managed perennial monoculture crop cultivated on large and small-scale. It is the chief plantation crop of the Darjeeling Hills and its adjoining plains (Terai and the Dooars). It is world famous for its quality (leaf tea) and excellent aroma. Districts of North Bengal provide a typical agro-climate for cultivation of tea on which several species of insect pests subsists. In Asia, 230 species of insects and mite pests are reported from tea plantations (Muraleedharan 1992). However, 173 arthropods and 16 nematodes are reported to be major and minor pests of tea in North East India (Hazarika 1994) including the plantations of Assam, which occupies a prominent position in Indian tea production (Sahewalla and Borthakur 1996). Among the insect pests, order Lepidoptera consists highest number of pest species (32%) followed by the order Hemiptera (27%) (Muraleedharan and Chen 1997). These pests are generally managed in conventional way by applying chemical pesticides. Extensive use of chemical pesticide has led to many well documented adverse consequences like environmental contamination, health hazard, pest resistance and death of natural enemy from the ecosystem (Ghosh Hajra 1994, Hajra 2002, Chattopadhyay et al. 2004, Obeidat et al. 2004). With long exposure to pesticides, resistant strains of insect emerge, requiring increased doses of insecticides and introduction of new insecticides. Therefore, the future protection and production of tea appear to depend largely on non-conventional control methods. For this reason biological pesticides are becoming key components of integrated pest management strategies (IPM) (Obeidat et al. 2004).

In many instances, alternative methods of pest control offer adequate levels of plant protection and management with fewer hazards.

In the present work an attempt to isolate and characterize naturally occucrring entomopathogenic bacteria from cadavers of three sporadic lepidopteran pests viz. *Arctornis submarginata*, *Andraca bipunctata* and *Orgyia postica* has been made. These pests occasionally attack the tea plantations of Darjeeling foothills, Terai and the Dooars as minor pests but sometimes the infestation crosses economic injury level (EIL). For collection of sporadic pest specimens (lepidopteran) and their cadavers, different tea plantations of the said regions were extensively surveyed. Isolation of bacteria, from dead larvae was done following techniques recommended by Lacey and Brooks (1997). After proving the Koch's postulates (Koch 1876, Fredericks and Relman 1996) the bacterial viability was checked by inoculating in new agar media. A total of nine *Bacillus* isolate were selected from three sporadic lepidopteran pest species (Arc01, Arc02, Arc03 from *A. submarginata*; Ab01, Ab02, Ab03, Ab04 from *A. bipunctata* and Org2A, Org6A from *O. postica*). Initial identification of the bacterial strain included microscopic examination like Gram's staining, crystal staining and endospore staining which established these bacterial isolates as *Bacillus* sp. Doubling time and biochemical tests were done to characterize the isolates. Sodium dodecyle sulphate Poly acralamide gel electrophoresis (SDS-PAGE) was done for the analysis of crystal protein and whole cell protein. The gels when analysed, Arc01, Arc03 and Ab04 showed 128, 122.7 and 129.8 kDa bands respectively, corresponding to ~130 kDa band *cry*1 gene. Result also showed that the strains Arc01, Arc03 had 64 and 56.3 kDa bands, respectively, while Ab01, Ab03, Ab04 and Org6A had bands of 56.7, 56.2, 56.5 and 57.7 kDa, respectively. All these bands correspond to ~60 kDa of *cry*2 gene. Plasmid profiles of all the nine isolates

were done. All the tests showed these newly isolated bacterial strains as comparable to the commercially available *Bacillus thuringiensis kurstaki* used as a standard but with a few differences which suggested that they may be novel isolates from this area. Next bioassay tests were done to evaluate virulence of these isolates against host insects. Here, three strains (Arc03, Ab04 and Org6A) showed highest toxicity to their respective host insects with LC_{50} values of 398.1, 486.6, 354.8 µg/ml, respectively. These most virulent strains were then sequenced for 16S rRNA gene, which in turn revealed a 99% similarity with the *Bacillus thuringiensis* strains available in the NCBI Genebank. When amplified for *cry* gene Arc03 showed presence of *cry*1 and *cry*9 genes, Org6A harbored only *cry*2 gene but Ab04 and *Btk* possessed all three *cry* genes i.e *cry*1, *cry*2 and *cry*9 genes. All these *cry* genes make them toxic to lepidopteran pests. Therefore it can be presumed that these isolates were infact, stains of *Bacillus thuringiensis* found naturally in these sporadic lepidopteran species of tea pests in the tea plantations of Darjeeling foothills, Terai and the Dooars. Further, counting on their virulence it may be taken for granted that these locally available strains of *Bacillus thuringiensis* may be developed as biopesticides in future.

Although importance of biopesticides as alternative pest management method is increasing day by day yet several constrains such as standardization and stable formulations, appropriate dispensing, ease of registration procedure of the new isolates and enhanced cost of application/ha etc. come in the way, specially in developing countries like India (Srinivasan 2012). It is hoped, that these newly reported strains of *Bacillus* with their appreciable entomopathogenicity would find their ways to get established as future biopesticides after meeting the said requirements.

PREFACE

Agriculture has a significant role in the socio-economic fabric of India. About 80% of Indian population depends on agriculture. Along with the development in agricultural techniques the problem related to pest has also evolved through time.

Century old tea cultivation has been economic backbone to about 70% of population of northern part of West Bengal. Tea, the foliage crop, cultivated as long-term monoculture provides an ambience for greater colonization by arthropods with maturation of the bush. Starting from root, stem maintenance and pluckable leaves, flowers and seeds are infested by wide range of pest species. A guild of defoliators that belong to the lepidopteran family has been reported to attack the plant regularly and sporadically causing havoc crop loss, hence require effective management.

Most of the tea planters of NE region employ the conventional method i.e. the use of pesticide to mitigate the pest related problem. Indiscriminate use of pesticides has caused serious concerns such as insect resistance to pesticides, the resurgence of pests, outbreak of secondary pests, harmful effects on human health and the environment which may lead to biological magnification in higher tropic level as well as can affect the non-target organisms directly.

One alternative eco-friendly approach to chemical pesticide is the use of microbial insecticides that include bio-agents such as bacteria, viruses, fungi, protozoa, and nematodes. The organisms used in microbial insecticides should essentially be non-toxic and non-pathogenic to wildlife, humans and other beneficial/industrial organisms as these are not closely related to the target pest.

Attempts has been made to develop the microbial pesticide for major lepidopteran tea pests has been carried out by earlier workers including one of my supervisor Professor Ananda Mukhopadhyay, continuing the trend of developing the same for sporadic lepidopteran pests, both my supervisors prompted me to undertake the study as Ph.D. work.

My Ph.D. work involved isolation and characterization of different entomopathogenic bacteria from three sporadic lepidopteran pests viz. *Arctornis submarginata* (Hairy caterpillar), *Andraca bipunctata* (Bunch caterpillar) and *Orgyia postica* (Sungma caterpillar) collected from tea gardens of Darjeeling foothills, Terai and the Dooars adopting polyphasic approach followed by testing virulence of these newly isolated bacterial strains against target pests through bioassays. Finally most virulent strains were amplified for 16s rRNA sequence for further identification, sequence were deposited in Gene Bank and the strains were screened for three lepidopteran toxic *cry* genes.

Overall outcome of the present research work will hopefully be able to furnish detailed information on development of potential bacterial pesticides against lepidopteran tea pests in future. As the results of the present work suggests that the bacterial strains (*Bacillus*) studied have the potential to be developed as microbial biopesticides in future with parallel effects as *Btk*, which is already in use for controlling different lepidopteran tea pests by reducing chemical contamination of environment.

Sangita Khewa Subba /11/17

ACKNOWLEDGMENTS

First and foremost, I would like to express my profound gratitude to my supervisors, Prof. Ananda Mukhopadhyay and Dr. Min Bahadur, of Department of Zoology, University of North Bengal, for their valuable and judicious guidance throughout the tenure of my Ph.D. research work. Their immense knowledge, enthusiasm, patience and supportive nature provided me a great opportunity to accomplish the objective of my research work.

My heartfelt thanks to Prof. Joydeb Pal, Prof. Tapas Kumar Chaudhuri and Prof. Sudip Barat former faculties of Department of Zoology. I extend my thanks to the Head, Dept of Zoology Prof. Soumen Bhattacharjee and all the facultiesof this department Dr. Dhiraj Saha, Mr. Tilak Saha, Dr. Saurav Mukherjee and Dr. Ritwik Mondal.
for their constant support and encouragement. Thanks are due to all the non-teaching staff of Zoology Department, UNB.

It is my privilege to express indebtedness and gratitude to my co-workers Dr. Damayanti De, Dr. Somnath Roy, Dr. Soma Das, Mr. Ritesh Biswa, Ms. Anjali Km. Prasad and Mr. Kumar Basnet for their friendly nature and their valuable suggestions not only in my research but also for the betterment of my life.

I am also thankful to my senior laboratory colleagues, of Entomology Research Unit for their immense support and valuable suggestions.

I wish to acknowledge my fellow scholars of the department, Dr. Rudra Prasad Roy, Mr. Binoy Kishore Rai, Mr. Pokhraj Guha, Mr. Dawa Bhutia, Ms. Ruksa Nur, Ms. Swati Singh, Ms. Sutanuka Chatraj, Ms. Uttara Bhowmik, Ms. Jayashree Soren, Mr. Mrinal Ray and others for extending their co-operation and support as and when needed.

Special thanks to Mr. Biswajeet Barman, Mr. Bhishmadev Barman and Mr. Tinku Roy for their constant technical support and always helping and relieving me during tiresome field work.

I am also thankful to the managers of various tea gardens for their cooperation and help during sampling.

I acknowledge the financial support from Rajiv Gandhi National Fellowship (UGC-RGNF), New Delhi and Council of Scientific & Industrial Research- Junior Research Fellowship (CSIR-JRF), New Delhi is duly acknowledged.

I also thank all my friends specially Ms. Swarna Dewan, Ms. Nimu Rai, Mr. Suchan Pradhan, Mr. Samik Chakravorthy, Mr. Kapil Tamang, Mr. Gunjan Rana, Mr. Thendup Lama, Mr. Sumendra Tamang, Mr. Prashid Rai, Ms. Nikita Lama, Ms. Ambika Rai, Ms. Priyashikha Rai for their encouragement and moral support throughout my Ph.D. Special thanks to my sisters Sumitra Tamang, Ms. Saloni Rai and Manisha Chettri for their help and support and Ms. Sneha Prasad for helping me in drawing maps.

Thanks are due to the Dr. Sujit Ghosh, Principal, Siliguri College, and my collegues Dr. Tanusree Biswas, Dr. Biplab Behera, Dr. Dipanjana Mazumdar and Dr. Sukanto Majumdar for their help and support.

Lastly, I woud like to acknowledge the encouragement and blessings of my parents Late Shree Shonam Khewa Subba and Smt Laxmi Khewa Subba, my better half Mr. Dhuniraj Chettri, my in-laws, Shree Deoraj Chettri and Smt Brinda Chettri and my elder sisters Mrs. Mamta Gurung, Mrs Ranju Gurung and Mrs Ramita Limboo my well-wishers and relatives for their moral support, love and blessings.

Date: **Sangita Khewa (Subba)**

Place:

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LIST OF ABBREVIATIONS

amsl	Above mean sea level
Btk	*Bacillus thuringiensis kurstaki*
°C	Degree Centigrade (=Celsius)
µg	Microgram
cm	Centimetres
Cs	*Camellia sinensis* (Tea)
Btk	*Bacillus thuringiensis kurstaki*
df	Degree of freedom
EDTA	Ethylenediaminetetraacetic Acid
EIL	Economic Injury Level
EPA	Environmental Protection Agency
EU	European union
F	Fisher
ft	Feet
g	Gram
h	Hour
ha	Hectare
ICP	Insecticidal Crystal Protein
IPM	Integrated Pest Management
kg	Kilogram
L:D	Light: dark phase
LC_{50}	Lethal concentration
LT_{50}	Lethal time
M	Mole/ Molar
m	Metre
mg	Milligram
min	Minute
MRL	Maximum Residue Limit
n	Number of observation
NaOH	Sodium hydroxide
NE	North-East

nM	Nanomolar
NPV	Nucleopolyhedrovirus
OD	Optical Density
p	Probability
PAGE	Poly acrylamide gel electrophoresis
pm	Picomoles
RH	Relative Humidity
SDS	Sodium dodecyle sulphate
SE	Standard error
w/v	Weight/volume

LIST OF APPENDICES

1. INTRODUCTION

1.1 Tea growing areas of the world and India

Tea, *Camellia sinensis* (L.) O. Kuntze, is an intensively managed perennial monoculture crop cultivated on large and small-scale between latitudes 41ºN and 16ºS. It is grown as plantation on over 2.71 million hectares (ha) in more than 34 countries across Asia, Africa, Latin America, and Oceania (Hazarika et al. 2009, Roy et al. 2015) (Fig. 1.1) to produce 3.22 million metric tons of made tea annually (FAO 2005). Due to its increasing demand, tea is considered to be one of the major beverages in the global market. As per the research study, in 2013, the global tea market was of US$38.84 bn and its dimension is projected to reach US$47.20 bn by the end of 2020. The market is expected to exhibit a 2.80% compound annual growth rate (CAGR) between 2014 and 2020 (Anonymous 2016a). The Indian tea industry flourished after a British national named Robert Bruce discovered indigenous tea plants for the first time growing in the upper Brahmaputra valley in Assam and adjoining areas in 1823 (Sivanesan, 2013). Since independence, tea production has grown over 250%, while land area has just grown by 40%. However, most of the tea bushes in Darjeeling have their origin from China "smuggled" out by a Scot botanist and adventurer Robert Fortune (Lama, 2013). In India, the crop is grown in the certain districts located in Assam, West Bengal, Kerala, Karnataka and Tamil Nadu and in a limited area of Tripura, Uttarakhand, Uttar Pradesh and Himachal Pradesh. (Fig. 1.2). The total area under tea in our country is about 5,63000 ha which produces 1208.78 million kg made tea annually (Annonymous 2014a). 76.3% of Indian tea is harvested from North East India In 2010, 43% of total country's tea production found place in the international export market (Bordoloi 2012)..

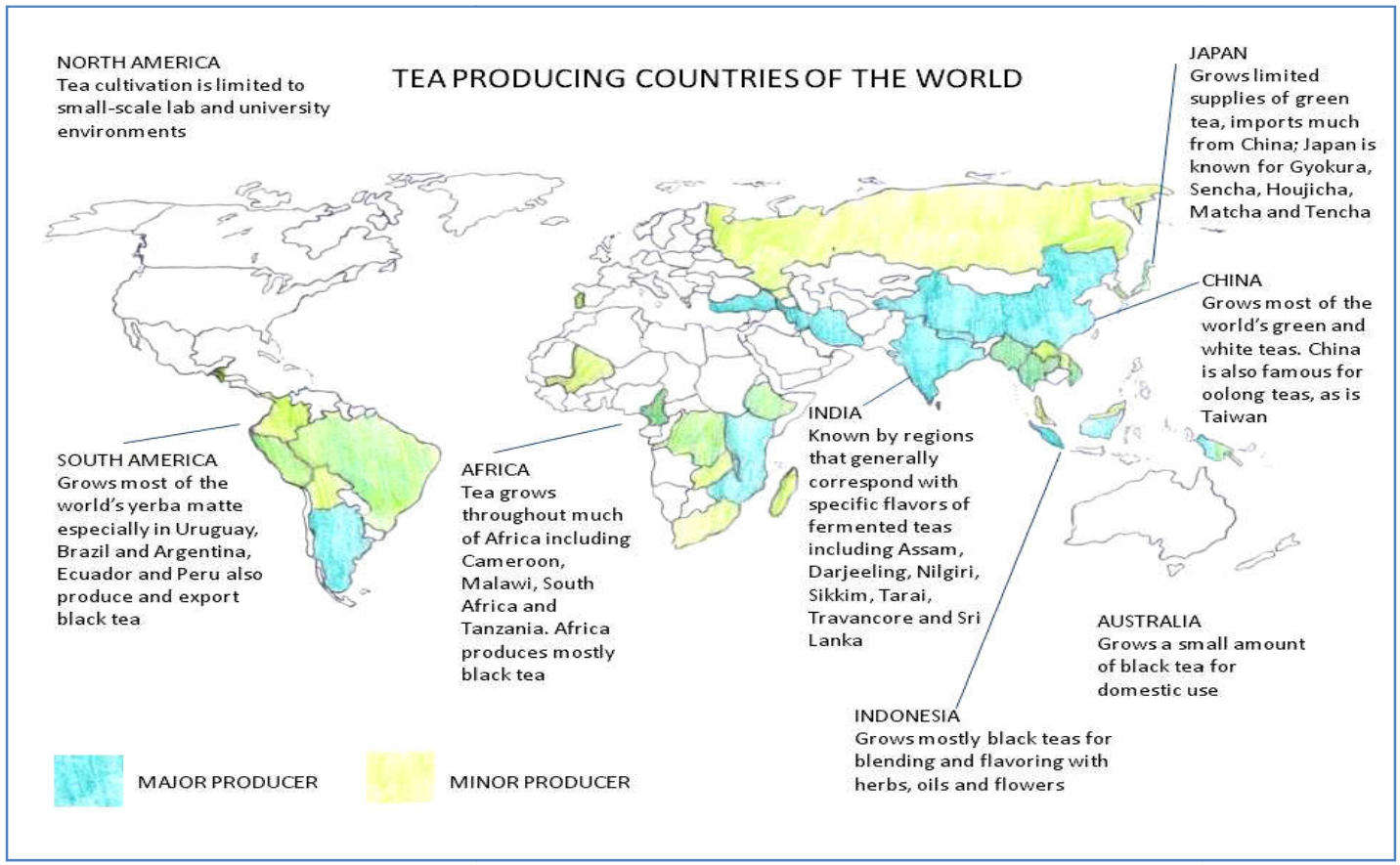


Fig. 1.1: Tea producing countries across the globe.

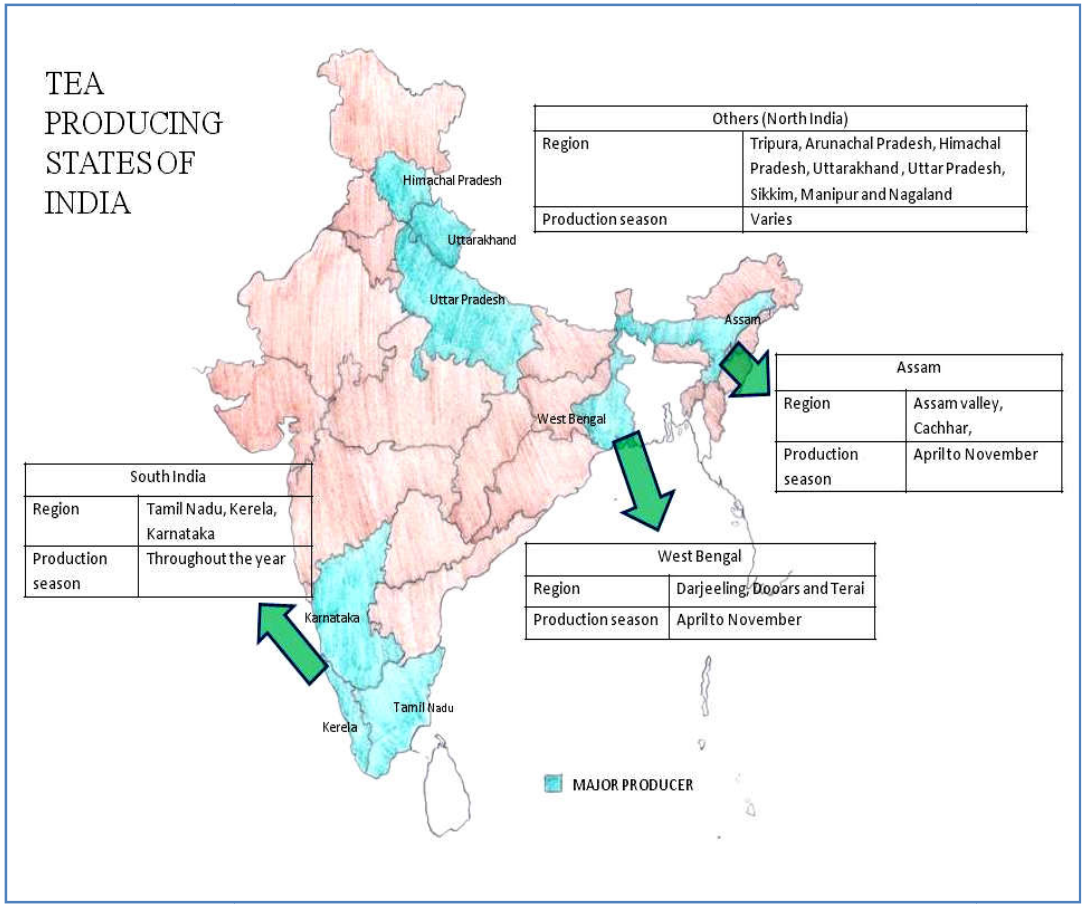


Fig. 1.2: Major tea producing states of India.

India is second largest producer contributing 24.46% to the world production and fourth largest exporter of tea (Annonymous 2014a, 2015).

1.2 Tea producing districts of West Bengal: Darjeeling foothills, Terai (Darjeeling) and the Dooars (Jalpaiguri, Alipurduar)

West Bengal offers teas from Darjeeling, Dooars and Terai and the Darjeeling is known as "The Champagne of Teas", cultivated on the slope of the Himalayas, have unique, delicate flavor and character (Arya, 2013). The tea growing regions of Darjeeling districts are located between 26°31′ and 27°13′North latitude and 87°59′ to 88°53′ East longitude. The northern part of the districts has the distribution of eastern Himalayas while the southern part consists of a stretch of alluvial plain at the base of the hills known as the Terai-Dooars. Darjeeling terai is situated at 91amsl with an average temperature of maximum 35°C and minimum 12°C. The Dooars or *Duars* (=Doors) area comprises flood plains and the foothills of the eastern Himalayas that continues in North East India and is the gateway to Bhutan. The altitude of the Dooars region ranges from 90 amsl to 1750 amsl. There are 308 big and 1232 small tea gardens in North Bengal region and the total area under tea is 122620 ha (Fig. 1.3). North Bengal region of Terai and the Dooars produces 25% of total tea of India (retrieved from www.teaboard.gov.in). In 2003, total production of made tea was 200 million kg (Anonymous 2003a). There are 87 functional tea gardens on a total area of 17,820 ha producing famous 'Darjeeling tea'. The total annual production is 8.91 million kgs. The Terai regions have an area of 49700 ha and in the Dooars region 72,920 hectares under tea plantations producing 125.34 and 177.84 million kgs of tea, respectively (Anonymous 2014a). As per the Tea Board data, the total tea production

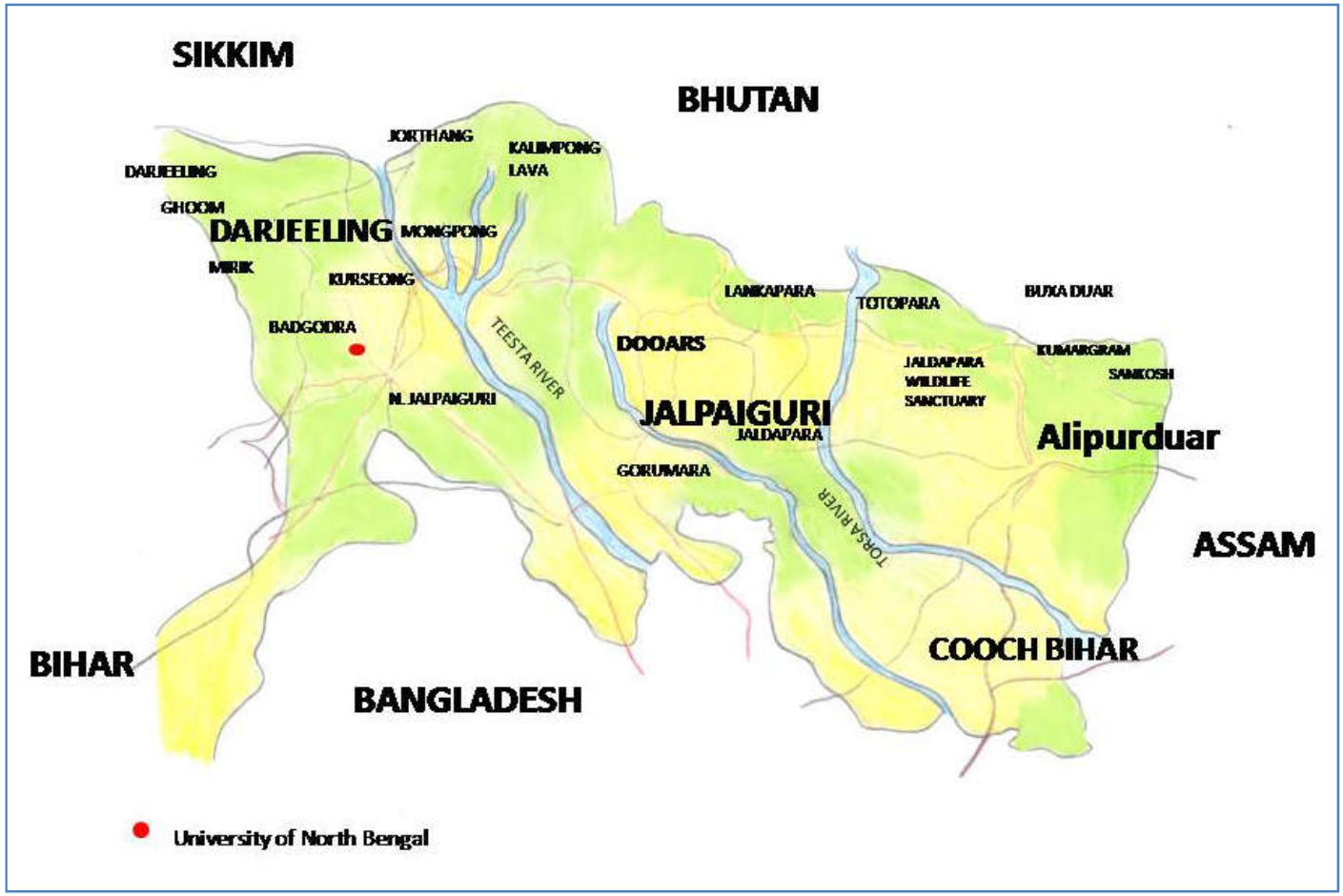


Fig. 1.3: Tea producing districts in Northern regions of West Bengal (North Bengal).

of the country was 1,233.14 million kg in 2016 against 1,197.18 million kg in 2015, 3% rise mainly because of good production in North India (Anonymous 2016b).

1.3 Pest Attack and Crop Losses

Tea plantation as monoculture is a permanent ecosystem which provides habitat continuity. Such an inexhaustible food resource can be colonized by for 1031 species of arthropods and 82 species of nematodes as reported from different parts of the world (Chen and Chen 1989). In Asia, 230 species of insects and mite pests attack tea (Muraleedharan 1992). However, 173 arthropods and 16 nematodes were reported to be major and minor pests of tea in North East India (Hazarika 1994) including Assam, which occupies a prominent position in Indian tea production (Sahewalla and Borthakur 1996). Among the insect pests, order Lepidoptera consists of highest number of pest species (32%) followed by order Hemiptera (27%) (Muraleedharan and Chen 1997). As insects have the dynamic ability to adapt; they can feed on various parts of the tea plant such as leaf, stem, root, flower and seed causing about 10 % loss in yield, which is generally accepted but, it could go up to 40% in case of devastating attacks by defoliating lepidopterans (Banerjee 1983). To control these pests, huge amount of pesticides is needed. Therefore, per hectare consumption of pesticides is often excessively high and also expensive (Chakravartee and Hazarika, 1995).

Looper caterpillars of, *Biston* (=*Buzura*) *suppresseria*, *Hyposidra talaca*, *Hyposidra infixaria*, *Ascotis* sp., *Ectropis* sp. (Lepidoptera: Geometridae) are found throughout the year and are the most destructive pest of tea plantations (Anonymous 1994).

B. suppressaria Guen was reported as a major tea pest in 1900 (Das 1965). They feed on the young leaves. Red slug caterpillar (*Eterusia aedea*) (Lepidoptera: Zygaenidae)

feed on the mature leaves of the tea bushes and the bark of young stem (Anonymous 1994). *Euproctis latisfascia* (Lepidoptera: Lymantriidae) feed on the mature and senescent leaves of tea bushes (Anonymous 1994). Flush worm (*Lespeyrasia leucostoma*) (Lepidoptera: Tortricidae) attacks on the pluck-able shoots and leads to the formation of a nest like structure due to folding of leaves. Sporadic infestation of defoliating Bunch caterpillar (*Andraca bipunctata*) (Lepidoptera: Bombycidae), mainly occurs during March to November in Tea plantations of North East India (Das 1965). Sporadic pests are defined as a species whose numbers are usually controlled by biotic and abiotic factors which occasionally break down, allowing the pest to exceed its economic injury threshold (Cherrett and Sagar, 1977, Hill 1983). Another sporadic pest, *Arctonis submarginata* (Lepidoptera: Lymantridae) has been reported relatively recently from the Darjeeling hills and Terai as an emerging tea pest feeding on the mature leaves of the bushes (Mukhopadhyay 2007). Sporadic and occasional attack by *Orgyia* sp. is also well known in the tea gardens of Darjeeling foothills and Terai. However, this pest was first recorded in a number of tea estates of central and western Dooars and Darjeeling during February- September (Das and Roy 1982). Another defoliator, *Orgyia postica* (Walker) (Lepidoptera: Lymantridae) was recorded attacking tea in North East India by Watt and Mann in 1903.

1.4 Brief description of the sporadic pests of tea

- **Hairy caterpillar (*Arctornis submarginata*):**

The hairy caterpillar of *Arctornis submarginata* (Walker) (Lepidoptera: Lymantriidae) (Fig. 1.4A) is found to attack and defoliate mature tea in the foothills and Terai of Darjeeling Himalaya. The geographic distribution of this insect includes North East Himalaya, Borneo and Sumatra (Schintlmeister 1994). Swarms of caterpillars consume and defoliate the mature and maintenance leaves of the tea bushes (Fig.

1.4B), adversely affecting the tea yield in Darjeeling foothill region (Terai) (Mukhopadhyay 2007). They have also been found in good numbers in the Dooars tea plantations. There are six instars in the life cycle of *A. submarginata*. Moths are white with two black spots on the forewing (Fig. 1.4C & 1.4D). They are found mostly in the winter season from November to January.

- **Bunch caterpillar (*Andraca bipunctata*):**

The bunch caterpillar *Andraca bipunctata* Walker (Lepidoptera: Bombycidae) is the earliest known tea pest repoted from Taiwan in 1820. It was recorded as a very widely distributed pest occurring over almost all the districts in North East India (Watt and Mann 1903) including Sub-Himalayan tea gardens of Darjeeling in West Bengal (Andrews 1921, 1931, Hainsworth 1952, Das 1956, Banerjee 1983, Ghorai 1992, Panigrahi 1998, 1999). The bioecology of *A. bipunctata* greatly varies depending upon climatic conditions (Das 1965, Ghorai 1992). Its conspicuous colour and peculiar habit of congregation on the branches and fast pace of defoliating the tea bushes can hardly escape notice. The young caterpillars remain congregated on the under surface of the leaf. They feed on the epidermal tissues, then at the leaf margin and ultimately the entire leaf. From the 3rd instar, they form typical clusters (Fig. 1.5A) on the branch and feed on the leaves. After eating all the leaves of a bush they migrate to the next plant. As a result of stunted growth, these infested plants remain unproductive for nearly 2 years (Ghorai et al. 2010). When present in large numbers they cause heavy loss by defoliating the leaves and even stripping the bark (Fig. 1.5B) of the tea bush. The moths are brown and dull (Fig. 1.5C & 1.5D).

- **Red hairy (*Orgyia postica*):**

The genus *Orgyia* comprises of 65-70 species. Overall, they have spider like appearance and are highly polyphagous in nature. The Lymantrid moth *Orgyia* sp. has a distribution in high altitude tea plantations usually above 500 m in the Darjeeling hills. The sporadic occurrence, but havoc depredation by caterpillars of the moth has been reported from tea plantations of Darjeeling foothills (Pathak 2003). In North East India, *Orgyia postica* was identified as the common defoliator of tea leaf since, 1930s by Watt and Mann.

The larvae of *Orgyia postica* (Walker) (Lepidoptera: Lymantriidae), are covered with bristles and can irritate human skin. Although the larvae sporadically occur, they rapidly defoliate plants causing considerable damage. The female is silky brown having profuse hairs all over the body surface with vestigial wings. The male moths have normal wings (Fig. 1.6C). The female moths being wingless, release a sex pheromone in order to attract males, copulate and lay eggs on the cocoon from which they emerged. Eggs are laid in clusters, loosely covered with hairs on the lower surface of the leaves, at the axil and stems and sometimes on the outer surface of the cocoon (Fig. 1.6D). The newly hatched caterpillars are gregarious in habit and feed on the epidermal tissue of the lower surface of mature leaves. As a result of damage small holes are formed on the leaf surface (Fig. 1.6B). The full-grown caterpillars (Fig. 1.6A) and other immature stages of caterpillars start feeding from the margin of the leaf and are mostly found on the top hamper on the under-surface of leaves and also the branches (Anonymous 1994).

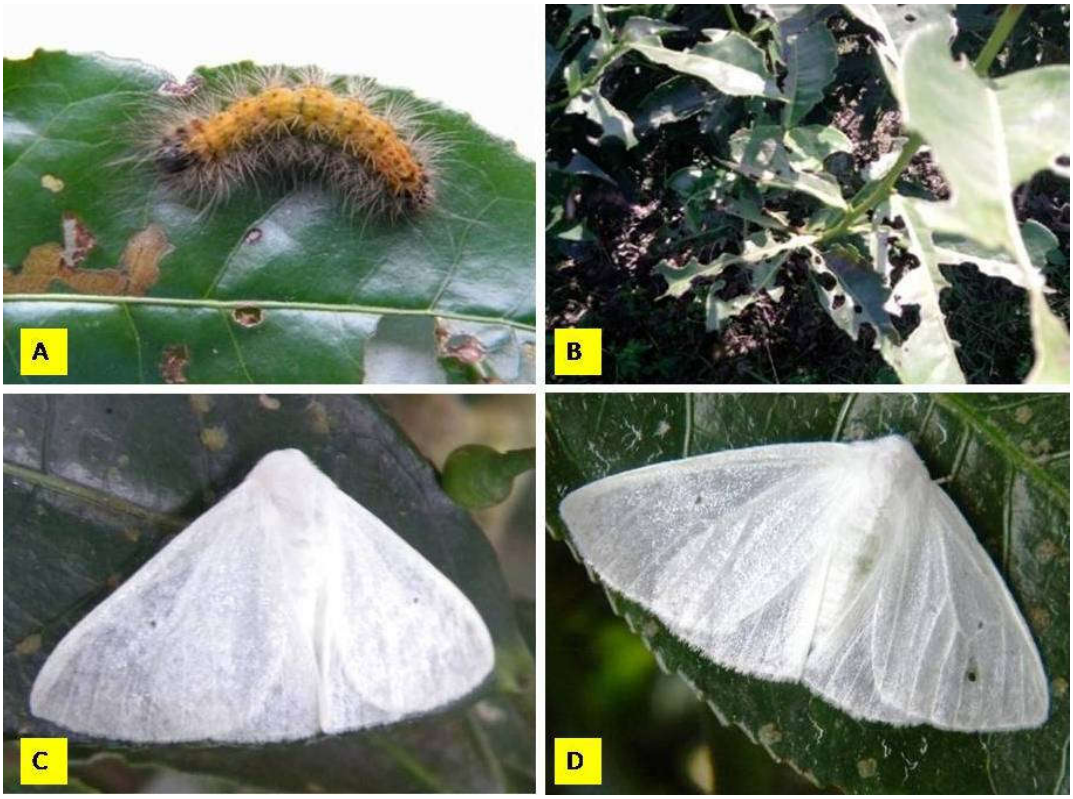


Fig. 1.4: A. Final stage caterpillar of *A. submarginata*; B. Damage symptom; C. Adult Male moth; D. Adult female moth.

Fig. 1.5A: Final stage caterpillar of *A. bipunctata*; B. Damage symptom; C. Adult Male moth; D. Adult female moth.

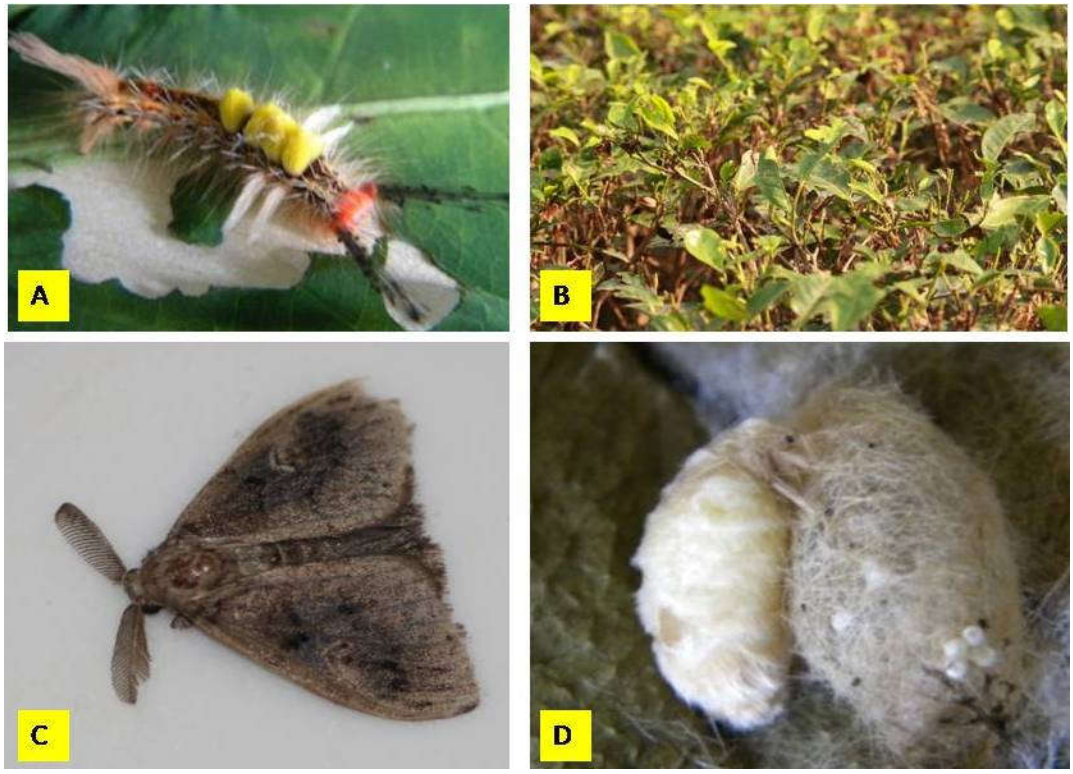


Fig. 1.6:A. Final stage caterpillar of *O. postica*; B. Damage symptom; C. Adult Male moth; D. Adult female moth (wingless).

1.5 Conventional methods of controlling the pest population

Pests, pathogens and weeds cause severe constraints to the productivity and quality of tea. In order to combat these, the tea planters use a wide range of pesticides to ensure high yield and economic returns. Because of the residual toxicity of pesticide in food products (Nagayama 1995, Neidert and Saschenbreker 1996) including made tea (Singh and Agnihotri 1984, Bishnu et al. 2009) there is an increasing public concern, of late, regarding the pesticide residues also in tea. Indiscriminate use of pesticides has caused serious concerns such as insect resistance to pesticides, the resurgence of pests, outbreak of secondary pests, harmful effects on human health and the environment (Muraleedharan and Selvasundaram 2005). Evidences also show that extensive use of chemical pesticide has had many well documented adverse consequences like death of natural enemy from the ecosystem (Ghosh Hajra 1994, Hajra 2002, Obeidat et al. 2004), resistant strains of insect pests, requiring increased doses of insecticides and introduction of new insecticides. Different chemical pesticides (Organophosphates and synthetic pyrithroids) have been found to be less effective against these defoliators (Sannigrahi and Talukdar 2003, Sarker and Mukhopadhyay 2006). Newer pesticides are highly expensive due to stringent safety standard national and International (Cooper and Dobson 2007). Moreover, health conscious consumers prefer organic tea to those of chemically managed conventional tea. Though broad spectrum plant protection chemicals offer powerful incentives for application, yet they have serious drawbacks including harmful effects on human health and the environment at large due to the presence of undesirable residues (Song et al. 1998).

1.6 Need of alternative methods to control these pests

The future protection and production of tea appear to depend largely on non-conventional control methods. For this reason biological pesticides are becoming key components in integrated pest management strategies (IPM) (Obeidat et al. 2004). In many instances, alternative methods of insect management offer adequate levels of pest control and pose fewer hazards. One such alternative eco-friendly approach is the use of microbial insecticides that include bioagents such as bacteria, viruses, fungi, protozoa, and nematodes. The organisms used in microbial insecticides should essentially be non-toxic and non-pathogenic to wildlife, humans and other beneficial/industrial organisms as these are not closely related to the target pest. The safety offered by microbial insecticides is their greatest strength. Its use has been largely increasing due to its high target specificity without harmful side effects (Fadel and Sabour 2002). There is a great diversity of these microorganisms infecting various insect pests. The entomopathogenic bacteria include species which can infect insects and multiply rapidly in the insect hosts following the infection (Aronson et al. 1986). The overuse or misuse of chemical pesticides and their negative impacts are increasingly becoming causes for concern, underlining the need for development of alternative pest control methods (Meadows 1993).

1.7 Importance of Microbial insecticides in controlling the pest population

Despite more than 10 fold increase in insecticide use since 1940 (Lysansky 1994), crop losses due to insects has nearly doubled in the same period. Therefore, microbial insecticides are especially valuable because their toxicity to non- target animals and humans is extremely low and moreover they do not pose adverse health effects even if

crops exposed to these microbial pesticides are consumed. Compared to other chemical insecticides, they are safe for both the pesticide user and consumers of treated crops (Mc Coy 1987). Microbial insecticides comprise of microscopic living organisms (viruses, bacteria, fungi, protozoan or nematodes) or the toxins produced. These bioagents function as pathogens of target insects causing infection leading to death of host insects (Charnley 1991). Of the various pathogens that attack insects and other invertebrates, insecticidal bacteria proved to be the easiest and most cost effective for mass production and thus were the first commercially successful microbial insecticides. The modes of action of these microbial agents to the given hosts differ depending on the organisms. Entomopathogenic viruses and bacteria must be ingested by the host insect along with their food for infection. In contrary entomopathogenic fungi may produce infection on contact by the reproductive propagules (spores or conidia) and also through ingestion by the host insect (Burges 1981). Microbial insecticides are relatively host specific, being adapted through co-evolution, to specific groups of insects, and will not cause infection in other groups of animals such as birds or mammals. But these microbes are very sensitive to biotic and abiotic factors of the environment (Lacey and Goettel 1995). Several decades of extensive investigations has resulted in broad use of microbial control in pest management (Burges 1981, Leggett 1995). It was in Japan in the year 1914 that the earliest efforts in microbial control of tea pest began (Hotta 1914). Microbial control measures are gradually being popularized in different tea-growing areas under integrated pest management for the production of chemical pesticide contamination free tea (Aizawa 1971, Kodomari 1993, Hazarika 2009, Ye et al. 2014). Various bacteria having the potential of biocontrol belonging to genus *Bacillus, Paenibacillus, Streptomyces, Pseudomonas* etc. were registered and approved as biopesticides

(Hynes and Boyetchko 2006). The tremendous success in microbial pesticides has come from the use of *B. thuringiensis* (Obeidat et al. 2004). *B. thuringiensis* strains show specific insecticidal activity against insects of different orders such as Lepidoptera, Coleoptera, Diptera, Hymenoptera, Homoptera, Orthoptera and Mallophaga (Schnepf et al. 1998). The most promising biological control agent *Bacillus thuringiensis* is the leading organism used as commercial microbial pesticides (Lambert 1992, Meadows 1993, Lysansky 1994). As of today, it is estimated that 95 percent of the market share of the pesticides is by bacteria in general and *Bacillus thuringiensis* Berliner (*Bt*) (Procaryotae: Firmicutes: Bacillaceae) in particular. It was reported that there was about 30 kinds of commercialized biopesticides globally during past decade (Xu 2008). The popularity of *Bacillus thuringiensis* product increased ever since 1990s. The sales were projected to more than double by the year 2000s (Bernhard 1993). In 1997, the global sales of *Bt* products was $ 984 million and in 2005 it went up to $ 3.6 billion (Wang 2006). Commercial *Bt* insecticides are Generally Regarded as Safe (GRAS) by the EPA, and are approved for most organic certification programs. Commercial preparations of *B. thuringiensis* (spore crystal mixture) have been registered as insecticides since 1961, and a significant quantity of toxicological data has been collected over recent years (Baum et al. 1999). However, *Serratia* sp. has been associated with insect disease (Klein and Jackson 1992) but commercial production of these bacteria was achieved only recently.

In the last few years, 59 pathogenic bacterial species have been developed as pesticides across the world. These various bacterial pathogens of insects are being used successfully in the biological control of insect pests (Thiery and Frachon 1997,

Sezen and Demirbağ 1999, Sezen et al. 2001). The microbial control of insect pests is of crucial importance in developing countries (González 1981).

Hence, the need for exploring and discovering entomopathogenic bacteria for management of pests has become essential, especially in organic farming to develop microbial pesticides from them in future. Integrated Pest Management in tea is greatly required (Barbora 1994) in NE India. One of the eco-friendly approaches of microbial control is conservation of the microbial bio-agents or their application for bringing in bacterial control of tea pests.

2 REVIEW OF LITERATURE

2.1 Synthetic insecticides: their merits and demerits in management of lepidopteran pests of tea plantation.

During the last six decades, the control of pests, diseases and weeds in tea fields is being predominantly done by the use of synthetic chemicals. In an average India uses of 0.5 kg ha^{-1} pesticide every year (Anonymous 2003a). The average use pattern of chemical pesticides was estimated to be 11.5 kg per ha in the Assam valley and Cachar, 16.75 kg per ha in the Dooars and Terai and 7.35 kg per ha in Darjeeling slopes (Barbora and Biswas 1996). The use of synthetic pesticides constituted 85% of the total pesticides used, in which, acaricides accounted for 25% (3.60 liter per ha) and insecticides 60% (8.46 liter per ha), while 15% were of organic and inorganic origin in the tea gardens of the Dooars (Roy et al. 2008). Among synthetic insecticides, organophosphates (64% - 5 rounds per year) were most preferred, followed by organochlorine (26% - 2 rounds per year) and synthetic pyrethroids (9% - 7 rounds per year) (Sannigrahi and Talukdar 2003). It has been estimated that the tea industry in India harbor about 300 species of insect pests (Das 1965) and therefore, extreme care must be exercised before a pesticide is introduced to tea for pest control to avoid residue build-up. Organophosphate, organochlorines, carbamate, synthetic pyrethroid insecticides have been in use in tea in NE India for the past 100 years. Much of the efficacy and sustainability of these groups of insecticides in tea pest management would depend on the susceptibility of the major target pests. An average of 7.5 lit of pesticides (quinolphos, acephate, monocrotophos, chlorpyriphos, cypermetrin and deltamethrin) per ha per year is applied to manage the pests in tea gardens of Terai and Dooars region (Roy et al. 2010). Bunch caterpillar, red slug and looper caterpillars are controlled by spraying profenophos /phosalone/ quinalphos in

early instars. But in late instar deltamethrin is used and lower part of the shade tree trunk is treated with insecticides in the case of red slug infestation. Organophosphates (profenophos and quinalphos) are highly effective against the larvae of tea looper in terms of time mortality, reduction in food consumption and nutritional indices, leaf area protection and preference index than synthetic pyrethroids, organochlorine and neonicotinoids (Bora et al. 2007). The ethion residue in Indian tea imported by countries of the European union (EU) was higher than the prescribed maximum residue limit (MRL) by 22.3%, 16.7% and 7.8% in Assam tea and 16.9%, 36.2% and Nil in Darjeeling tea in the year 2002, 2003 and 2004, respectively (Anonymous 2002, 2003b, 2004). Assam and Darjeeling tea continue to record high MRL values for organochlorine and synthetic pyrethroid residues, very few of which conformed to the EU maximum residue level. Use of Endosulfan (41.1 to 98.0%), Dicofol (0.0 - 82.4%) and Cypermethrin (6.0 - 45.1%) have remained comparatively high during 2002 to 2004 in different tea growing areas of NE India (Anonymous 2002, 2003b, 2004). Further, it is pointed out that impurity in dicofol, which contains DDT as contaminant might be causing the adverse residual effect. The EU after analyzing tea imported by them for residue contents (783 samples out of 6217 tea samples all over the world) have classified the Indian tea under "higher incidence of pesticide residue group". The MRL for most of the chemicals in the EU has been fixed at < 0.1 ppm., which has been a major constraint to tea exporting countries (Anonymous 2002, 2003b, 2004). Thus the demand for production of residue free tea is increasing in tea-exporting countries.

Samples taken from tea plantations in Darjeeling contained varying levels of residue in made tea. About 28% of 182 first flush samples and 31.5% of 89 second flush samples were found to carry residues above the MRLs. In another set of 65 samples of made tea,

43% contained ethion residues with a maximum of 8.43 ppm, and 18% of the samples contained dicofol residues of 6.4 ppm (Barooah 1994) that were much above the MRL standards prescribed by international agencies such as Environmental Protection Agency (EPA), CODEX, EU etc.

'Teekane,' the Darjeeling Gold brand of tea had been rejected from market by Germany as it contained 0.24 mg of tetradifon-a pesticide used against mites in tea which was 24 times the MRL fixed by Germany (http://www.nabard.org/roles/ms/ph/tea.htm). Heptachlor and Chlorpyrifos pesticides despite being banned are prevalent in made tea at higher concentration than their respective MRLs (Bishnu et al. 2009). Therefore, to restrict the extensive use of synthetic pesticides, Tea board of India has implemented 'Plant protection Code, ver.3' from 01/01/2015 (Anonymous 2014b). Synthetic pesticides also impose serious chemical stress to the environment often resulting in resurgence of primary pests (Sivapalan 1999), secondary pests outbreak (Cranham 1966), resistance development (Roy et al. 2010) and environmental contamination including undesirable residues in made tea (Chaudhuri 1999). Continuous use of synthetic pesticides is known to facilitate the development of higher tolerance or resistance in many insect (Martin et al. 2002, Komagata et al. 2010, Basnet et al. 2015), thus requiring even higher dose of pesticides. Though synthetic pesticide consumption in tea has gone up to 40.91% in 2004 as compared to 10.2% in 1998, reports of pest control failures are frequent (Gurusubramanian et al. 2008, Roy et al. 2010). At present it is a global concern to minimize chemical residue in food, including beverages, fruits and vegetables. Some countries have specified very low residue limits for certain chemicals in tea. Germany has specified 2 ppm levels for Ethion and Dicofol against 10 ppm and 45 ppm, respectively by EPA of USA (Barbora 1994). .

2.2 Entomopathogenic bacteria: their pathogenicity and potential for use as microbial pesticides

The entomopathogenic bacteria are considered to be much more selective and safer for humans and non-target organisms due to their narrow target group than most conventional synthetic insecticides. Pathogenicity is largely associated with entry of pathogen into the haemocoel of an insect either through a wound in the exoskeleton or more generally thorough the peritrophic membrane of the gut. Among the Gram-negative bacteria, some members of the family Enterobacteriaceae are recognised as good insect pathogens. *Serratia* sp. in particular, has often been associated with insect disease and a commercial product containing *S. entomophila* is being used to control the grass grub *Costelytra zealandica* in New Zealand. The bacteria turn the larvae to yellow or amber colour, hence the name "amber disease" (Jackson et al. 1992). Muratoglu (2009) found that *Pseudomonas putida* was effective in killing the larvae of Colorado potato beetle. Babu (2010) established the efficacy of *Pseudomonas fluorescens* against *Oligonychus coffeae* infesting tea. A year later, Roobakkumar (2011) showed that *P. fluorescens* produced bacterial chitinases responsible for killing the mites by hydrolyzing chitinous exoskeleton. The hazelnut leaf holer *Anoplus roboris* (Coleoptera: Curculionidae) a devastating pest of hazelnut and oak trees (Ecevit et al. 1993, Anonymous 1995) causes approximately 20-30% economic damage to hazelnut production per year in Turkey. In order to find a more effective and safe biological control agent against *A. roboris*, the bacterial flora of the hazelnut leaves holes were investigated and tested for insecticidal effects on it. According to morphological, physiological and biochemical tests, bacterial flora were identified as *Bacillus circulans* (Ar1), *Bacillus polymyxa* (Ar2), *Enterobacter* sp. (Ar3) and *Bacillus sphaericus* (Ar4). Generally hundreds of bacterial species have been found to be

associated with insects (Deacon, 1983). It is known that many bacteria which can be isolated from insects belong to genera *Bacillus* and *Enterobacter* (Tanada and Kaya 2012) among which some are pathogenic to the host.

The alder leaf beetle *Agelastica alni* (Coleoptera: Chrysomelidae) is another devastating pest of hazelnut and alder trees throughout the world (Suchy 1988, Baur 1991, Urban 1999, Sezen et al. 2004). Sezen et al. (2004) have identified *Enterobacter agglomerans* (Aa1), *Listeria* sp. (Aa2), *Pseudomonas chlororaphis* (Aa3) and *Pseudomonas fluorescens* (Aa4) as entomopathogenic to the above beetle. In another study Martin et al. (2008) found two strains of non-spore forming bacteria *Enterococcus faecalis* which were toxic to *Manduca sexta* larvae similar to the toxicity shown by *E. faecalis* against greater wax moth (Park et al. 2007). Forst and Nealson (1996) identified toxin complexes (tc) from *Photorhabdus luminescens* which acts on the midgut of the insect to kill it. Same kind of toxin complexes was later identified by a number of workers in other bacteria (Hurst et al. 2000, Morgan et al. 2001).

Serratia marcescens is found to be pathogenic to two species of scale insects of tea, *Paralepidosaphes tubulorum* and *Chrysomphalus ficus* (Wang et al. 2010).

The other important class of Gram-negative pathogens comprise the nematode-borne micro-organisms, *Photorhabus* and *Xenorhabus* that provide a fascinating story of symbiosis and pathogenicity. These closely-related members of the family *Enterobacteriaceae* are carried as symbionts in the intestine of the juvenile of certain nematodes. The nematodes infect insect larvae and upon entering the haemocoel release the bacteria which, together with the nematode kill the insect host. The bacteria release toxins which affect the larva and also provide nutrients for the

nematodes. During the later stages of the infection, the bacteria and nematodes re-associate to move on to new pastures (Boemare et al. 1997).

2.3 Entomopathogenic bacteria: categories and strains

The entomopathogenic bacteria have been divided into two groups, the spore formers (Gram-positive) and non spore formers (Gram-negative), which are further divided into obligate and facultative. The Gram-positive entomopathogenic bacteria have the advantage due to their ability to form spores during development which enables them to become resistant to environmental changes like temperature, humidity, etc. and allow them to persist in dormant condition outside the target host but proliferate rapidly after getting ingested by the host. Facultative spore-formers may be crystalliferous (crystal producing) or non-crystalliferous (Srivastava 2004). The classification of entomopathogenic bacteria has been presented schematically in the Fig. 2.1.

The crystalliferous spore forming bacteria are seen to be more virulent than their non-crystalliferous counterparts, the reason being the presence of crystal protein which is highly toxic to the insect host (Prieto-Samsonov 1997). Although these Gram-negative organisms have their applications in insect control, the Gram-positive bacteria have proven to be the most useful pathogens for biological control purposes and form the basis of the microbiological insecticide industry. One of the extensively studied spore forming crystalliferous entomopathogenic bacteria is *Bacillus thuringiensis* (*Bt*) which was brought to notice for its excellent control over the insect pest as early as in 1950s (Steinhaus 1956).

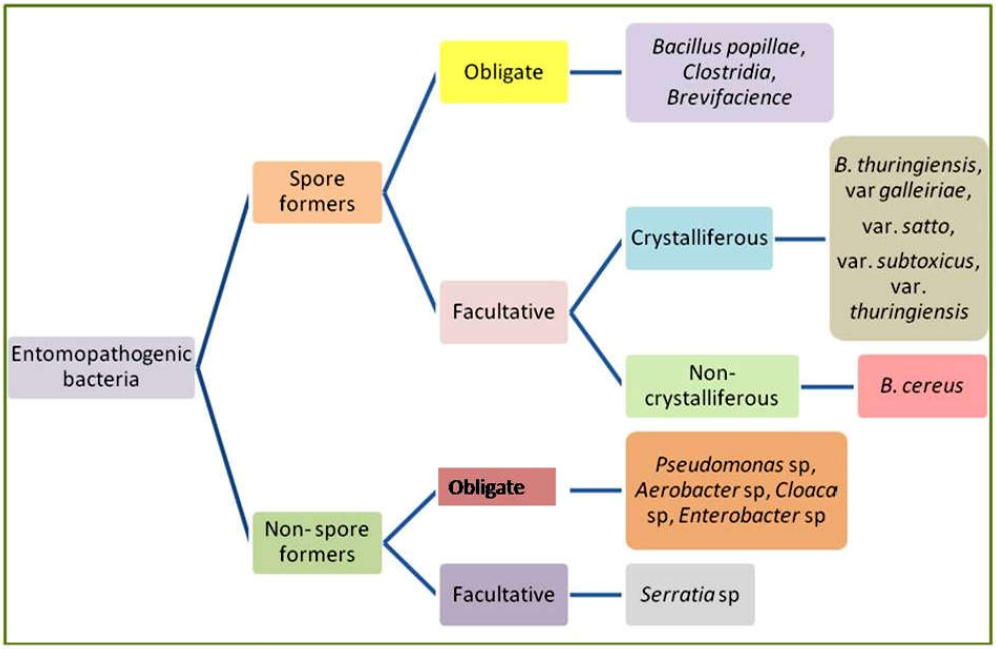


Fig. 2.1: The classification of entomopathogenic bacteria

Common caterpillar pests that are controlled effectively with *Bacillus thuringiensis* var *Kurstaki* include European corn borer, Indianmeal moth, Cabbage Looper, Imported cabbage worm, Diamond back moth, Gypsy moth, Spruce budworm, Tomato/ Tobacco moth etc. (Brownbridge 1991, Weinzierl et al. 1998). In India, the pod boring lepidopteran pests are major insect pests of *Cajanus cajan* (Pigeon pea) (Lateef and Reed 1983, Reed and Lateef 1990). These pests could be to a large extent controlled by application of *Bacillus thuringiensis* Berliner *Kurstaki* (Putambekar et al. 1997). Different *Bt* subspecies has been continuously tested for control of *Spodoptera litura* (Lepidoptera: Noctuidae) (Amonkar et al. 1985, Devi et al. 1996, Kamala Jayanthi and Padmavathamma 1997). Moreover *Bt* has been found to be at par and sometimes more effective than chemical insecticides (Malathi et al. 1999, Gupta et al. 2000). When efficacy of two subspecies of *Bacillus thuringiensis* (*B.t aizawai* and *B.t kurstaki*) was tested on *S. Litura*, it was found that the former was more virulent than the latter because of its higher viable spore count and total protein content (Pandey et al. 2009).

2.4 *Bacillus thuringiensis* as an efficient biopesticide against various tea pests

2.4.1 *Bacillus thuringiensis*: a historical perspective

The bacterium, that later became known as *B. thuringiensis* was first discovered by Ishiwata (1901) in Japan during his study of a bacterial disease of silkworm. His study of the sotto or sudden death *Bacillus* was published in Japanese therefore, it was unknown to researchers outside Japan for a long time. Fourteen years later, in 1915 in Germany Berliner identified a pathogenic *Bacillus* killing the larvae of flour moths and subsequently published description of the bacterium and its properties, naming it

Bacillus thuringiensis Berliner (Berliner 1915). Hannay (1953) and Angus (1954) proved that the parasporal crystals present in *Bacillus thuringiensis* were responsible of killing silkworms. The active spectra of insecticidal crystal proteins (ICPs) includes larvae of Lepidoptera, Coleoptera, Diptera, Hymenoptera, Homoptera, Mallophaga and Orthoptera (López-Pazos and Cerón 2007). The history of development of *Bt* as microbial control agent is presented in the work of Beegle and Yamamoto (1992) after which many isolates of bacteria with specific activity were commercially produced.

2.4.2 Natural occurrence of *B. thuringiensis*

Numerous *Bacillus thuringiensis* (*Bt*) species have been isolated from dead or moribund insect larvae and in most cases the isolate has toxic activity to the insect from which it was isolated (Goldberg and Margalit 1977, Burges 1981, deBarjac 1981, Hansen et al. 1996). Numerous *Bt* subspecies have been recovered from coniferous trees, deciduous trees and vegetables, as well as from other herbs (Damgaard 1997, Smith and Couche 1991). The *Bt* isolates have demonstrated a broad diversity both with specific activities to insects from the orders Coleoptera and Lepidoptera (Damgaard 1997, Hansen 1998). *Bt* has been found extensively in the phylloplane. A total of 120 *Bt* strains were isolated from 35 species of phylloplanes both trees and shrubs in a relatively small area near Tokyo by Evans (2002). The spores of *Bt* persist in soil and vegetative growth occurs when nutrients are available (DeLucca. et al. 1981, Travers et al. 1987). DeLucca. et al. (1981) found that *Bt* represented 0.005 to 0.5% of all *Bacillus* species isolated from soil samples in the USA. Martin and Travers (1989) recovered *Bt* from soils globally. Meadows (1993) isolated 785 *Bt* strains out of 1115 soil samples. Bravo et al. (1998) have characterized *cry* gene in Maxican *Bacillus thuringiensis* strains collected from soil

samples. Reports of *Bt* from fresh water (Ichimatsu 2000), ware house (Hongyu 2000), compost (Bernhard 1997), gastroenteritis outbreak (Jackson et al. 1995), marine sediments (Maeda et al. 2000), animal feed mill (Meadows et al. 1992), live stock ecto-parasites (Gough et al. 2002), dairy, human pus, nose, eyes, urine (Helgason 2000), Antarctica (Forsyth and Logan 2000), ancient glacial ice (Christner et al. 2003) Mount Everest (Shrestha et al. 2007) further confirms the ubiquitous presence of a wide variety of *Bt* isolates. Therefore it is evident that *B. thuringiensis* strains are distributed worldwide, which needs to be characterized to evaluate their toxicity against different orders of pest species (Chak et al.1994, Theunis et al. 1998, Bravo et al. 1998, Forsyth and logan 2000, Uribe et al. 2003).

2.4.3 Mode of action on target insects

The bacterium produces insecticidal crystalline inclusions popularly called δ-endotoxins, mainly during the late exponential phase and stationary phase of growth. These δ-endotoxins (Cry proteins) are toxic to various organisms. To activate the protoxins a susceptible insect must eat them. For most lepidopterans, protoxins are solubilized under the alkaline conditions of the insect midgut (Hofmann et al. 1988). The active toxin binds specifically to the brush border membrane vesicles of the midgut epithelium cells, leading to osmotic imbalance (Spore germination and proliferation of the vegetative cells into the haemocoel) and finally the insect dies of septicemia. Receptor binding by the insecticidal crystal protein (ICP) is the major determinant of host specificity by the different *Bt* ICPs. The efficacy of the ICP depends on the solubilization in the midgut. Differences in the degree of solubilization sometimes explain differences in the degree of toxicity among Cry proteins (Du et al. 1994). Many *B. thuringiensis* strains which have wider spectrums

of insecticidal activity express several kinds of crystal protein (Lee et al. 2001). The mode of action and host specificity of Cry toxins have been reviewed by different workers (Bravo 2005, Bravo et al. 2007, Pigott and Ellar 2007).

2.5 *Bacillus thuringiensis* as biocontrol in tea pests

Bacillus thuringiensis constitutes the most widely used biological insecticides (Gawron-Burke and Baum 1991). The main feature of the Gram-positive spore forming bacterium *Bacillus thuringiensis* is the production of proteinaceous crystalline inclusions (crystals) during sporulation, which are responsible for its toxicity towards a variety of invertebrates, especially insects (Padidam 1992, Bravo et al. 2007, Sauka and Benintende 2008).

Cry proteins are classified according to their amino acid similarity in 59 major groups divided into different classes and subclasses (Reyaz, 2016).

Wide range of *Bt* insecticides are being formulated from *Bacillus thuringiensis* var *Kurstaki* which are toxic only to specific order of insect. It accounts for more than 90% of the biopesticides used today (Feitelson et al. 1992, Koul 2011). Use of bacteria especially *Bt* is well established as microbial pesticide of different tea pests (Kariya 1977, Barbora 1995, Hazarika et al. 2008). Pest control is essential to stabilize tea production, because there are many kinds of pests with large populations that cause serious damage to tea plants. *B. thuringiensis* produces a toxin, which shows its toxicity in the body of larvae of lepidopteran insects and kills them. The killing effect differs with different strains of the *Bacillus* (Ebihara 1972, Kusuno 1973) and with different kinds of insect (Kusuno 1975).

B. thuringiensis was used in China to control the lepidopteran pests of tea and it was successful in controlling 95% of the pest (Yu and Lin 2008). Wu (1981) isolated and

identified a strain from larval cadavers of *B. supressaria* which was named *B. thuringiensis* var. *finitimus* strain CW-1. Similarly, Tan and Lu (1985) isolated three *Bt* strains (strains 111, 119 and 109) from *Euproctis pseudoconspersa* cadavers, of which strains 111 and 119, caused 74% mortality of the larvae. In Japan, formulations of *Bacillus thuringiensis* are reported to be effective in controlling the oriental tea tortrix, *Homona magnanima* and are thus being used in the Integrated Pest Management (IPM) of this pest (Kariya 1977). Many reviews on bacterial pathogens of tea pests (Hazarika 1994, Agnihothrudu 1999, Hazarika 2009) lead to the fact that almost all entomopathogenic bacteria isolated from tea pests are *Bacillus thuringiensis* (*Bt*) (Borthakur 1986, Ghosh Hajra 1994, Hazarika 1994, Barthakur 2003, Barthakur 2011). *B. thuringiensis* sub sp. *thuringiensis* strain HB III was used to control *B. suppressaria* in India by Borthakur and Raghunathan (1987). Mukherjee and Singh (1993) also tested *Btk* to control the looper pest. Their study also showed that at sub lethal dosages *B. thuringiensis* could arrest the feeding of *B. suppressaria* which could be effectively used to minimize crop loss in tea. Gurusubramanian (2008) reported that *B. thuringiensis* var *kurstaki* resulted significant mortality of 45-95% against *B. suppressaria, Andraca bipunctata* and *Scirtothrips dorsalis* in North East India. The control effect of *Bt* on tea pests *Euproctis pseudoconspersa*, *Ectropis obliqua* and *Andraca bipunctata* reached above 90%, and is safe for natural enemies (LingLing et al. 2004). It is reported that *B. thuringiensis* formulations have been applied efficiently against tea pests, such as *Caloptelia theivora* (Unnamalai and Vaithilingam 1995).

Commercial formulations of *Bacillus thuringiensis* like Dipel were also used for the control of tortricid caterpillars in the tea fields of Japan (Kodomari 1993). Barbora (1995) emphasized the importance of such formulations in tea pest management in

India based on laboratory bioassays on the looper and bunch caterpillars. Many studies on *B. thuringiensis* formulations to improve its persistence and field efficacy are evident (Cranham 1966, Kodomari 1993, Hazarika et al. 2005). Singha (2010) treated two termite species of tea garden *Microterms obesi* and *Microcerotermes beesoni* with *Bacillus thuringiensis* and found astonishing 80% mortality.

2.6 Resistance to *Bacillus thuringiensis*

Resistance to commercially available *Bt* occurs when there is a secondary outbreak of more damaging (Hoy 1998) and genetically variant pests, which are not susceptible to the *Bt* delta endotoxins. These resistant individuals are unaffected by the *Bt* toxins since they possess variant forms of receptor molecules or are equipped with a mechanism to break down these toxins (Michaud 1997). *Bt* resistance was first discovered in *Plodia interpunctella* (Lepidoptera: Pyralidae) in 1985. Various other insect species have been shown to develop resistance to *Bt* toxin in the laboratory, including *Ostrinia nubilalis* (the European corn borer), *Heliothis virescens* (the tobacco budworm), *Pectonophora gossypiella* (the pink bollworm moth), *Culex quinquefasciatus* (mosquito), *Aedes aegypti* (the yellow fever mosquito), *Trichloroplusia ni* (the tiger moth), *Leptinotarsa decemlimeata* (the Colorado potato beetle), *Spodoptera exigua* (the beet armyworm), *Spodoptera littura* (the Egyptian cotton leaf worm), and *Chryosomela scripta* (the cottonwood leaf beetle) (Tabashnik 1994, Liu et al. 1998, Wirth 1998, Frutos 1999).

The rate at which insects develop resistance depends on the reproductive rate of the species, their generation time, number of progeny, and the time of exposure to the toxins. The resistance is developed more rapidly in insect species that have higher reproduction rates and greater number of progeny (Whalon and Norris 1996). There are various mechanisms by which resistance develops in the insect. For instance in

Plutela xylostella there is a change in the structure of the membrane receptors (Tabashnik 1994), which affects the toxin-receptor affinity, resulting in less toxin molecule binding and a 100-fold decrease in toxicity (Van Rie 1990a,b). Moreover, there is an absence of a major gut proteinase in *P. xylostella* (Oppert 1997) which is correlated with decreased activation of *Bt* Cry toxins. *Heliothis virescens* also shows evidence of developed resistance by the decrease in activity of protoxins and reduced binding of the toxins to their complementary receptor-binding sites (Parker and Pattus 1993). It is still necessary to search for more strains and toxins, since a significant number of pests are not controlled with the available *Bt* strains. It is also important to provide alternatives for insect resistance, especially with regard to the transgenic crops (Bravo et al. 1998).

2.7 Need for searching novel and improved entomopathogenic bacterial strains

The demand for organic products and the development of resistance of insect to conventional pesticide as well as biological pesticides (Gujar and Kalia 1999, Griffitts et al. 2005) have led researchers to search for additional environmental bacteria that kills pests. Moreover in recent years the importing countries are imposing stringent restriction standards as regard to the MRL in made tea (Roy et al. 2011). These insecticides are highly specific to particular insect gut receptor, therefore not harmful to the non-target organisms (Lacey and Mulla 1990, Melin and Cozzi 1990, Glare 2000, Lacey and Siegel 2000) including vertebrates (Laird et al. 1990, Saik et al. 1990). For these reasons there is currently great interest in isolating strains of *Bt* with either host specificity or elevated toxicity. Therefore, agrochemical and pharmaceutical corporations have already initiated intensive research programs to

isolate Bt from various environmental samples and to evaluate their toxicity in agriculturally and medically-important target pests (Van Frankenhuyzen 1993).

As *Bt* is present in every habitat possible in this earth, therefore each habitat may contain a novel *Bt* strain awaiting discovery which has a toxic effect on a target insect group (Apaydin et al. 2005). The characterization of native *B. thuringiensis* strains helps in understanding the role of bacteria in the native environment and distribution of *cry* genes in local conditions (Ben-Dov et al. 1997, Bernhard et al. 1997). To increase the available toxin gene pool, extensive strain search and assessment programmes were undertaken (Martin and Travers 1989, Meadows 1993, Bernhard 1997). Report of local *Bt* strains from all over the world is available *viz.* Iran (Keshavarzi 2008, Aramideh et al. 2010), Bangladesh (Shishir et al. 2012b, Shishir et al. 2014), Turkey (Demir et al. 2002, Sezen et al. 2004, Apaydin et al. 2005, Kati et al. 2007, Ozturk et al. 2008), Vietnam (Binh et al. 2007), Korea (Lee et al. 2001), Sri Lanka (Zakeel et al. 2010), Trinidad (Rampersad and Ammons 2005), Latin America (Ibarra et al. 2003), Brazil (Monnerat 2005), Canada (Cardinal and Marotte 1987) and Japan (Ohba et al. 1987).

Additionally, *Bt* toxins are biodegradable and do not persist in the environment (Van Frankenhuyzen 1993). More than 50,000 *Bt* strains isolated by screening procedures are distributed among various private and public collections. These are considered to be potential reservoirs of novel toxins (Ohba 1996, Sanchis et al. 1996). *B. thuringiensis* has been proved to be an effective pesticide in horticulture and forestry (Keller and Langenbruch 1993, Teakle 1994) and in controlling the medically important insects such as mosquitoes and black flies (Ritchie 1993, Becker 1997). Studies by different workers have shown that *Bt* insecticides have no mammalian

toxicity (Siegel 2001) and also toxic volatiles are not released during or after spraying the insecticide (Van Netten et al. 2000).

Some naturally occurring entomopathogenic *Bacillus* have been isolated form Terai tea plantations of Darjeeling foothill regions which were found to be effective against number of tea pests like tea loopers *Biston* (=*Buzura*) *suppressaria, Hyposidra talaca,* red slug caterpillar *Eterusia aedea*, leaf roller caterpillar *Caloptilia theivora* and the hairy caterpillar, *Arctornis submarginata* (De 2007, De and Mukhopadhyay 2008, 2010, 2011, Khewa and Mukhopadhyay 2010, 2012, Mukhopadhyay et al. 2010, Khewa et al. 2014). Infected larvae of *B. suppressaria*, *H. talaca*, *E. aedea*, *C. theivora* and *A. submarginata* yielded spore forming bacteria with crystal proteins and appeared to share many features in common with *Bacillus thuringiensis kurstaki* (*Btk*). A virulent but non-spore forming bacterial pathogen (*Enterobacter* sp) was also reported from *C. theivora* by De et al. (2008).

Until the early 1980s, commercial *Bt* products were effective only against caterpillars. In recent years, however, additional isolates that kill other types of pests have been identified and developed for insecticidal use (Weinzierl et al. 1998). Once such integrated pest management modules are developed using appropriate control techniques including microbial control in a mutually reinforcing manner, a check on the tea pest populations to a non-damaging level can be easily obtained. Entomopathogenic bacteria hold a great promise of their future applications in tea pest management as biocontrol agents especially in the organically produced export quality Darjeeling tea. Consequently, they are likely to become increasingly important tools in insect pest management.

2.8 Importance of Plasmid profiling in characterization of *Bacillus* strains

Plasmid DNA has been considered as one of the most important tools in biotechnology, agriculture, molecular biology and bio control (Simeon et al. 2003). Variations in the number and molecular weight of the plasmid DNA represent the genetic divergence between the strains of that species. Plasmid patterns have frequently been used to characterize strains (Ibarra and Federici 1986, Ibarra et al. 2003). *Bt* plasmids have been studied either to locate *cry* genes or to transfer them to different strains and species. Reyes-Ramírez and Ibarra (2008) studied plasmid patterns of several strains of *Bt* and observed that, all strains except one showed a unique plasmid pattern.

2.9 Importance of 16S rRNA sequencing for identification of novel bacteria

Reclassification and renaming of numerous bacteria have been possible because of 16S rRNA sequencing. Identification of uncultivable bacteria has been made possible, phylogenetic relationship has been established and discovery and classification of new bacterial species have been made easy. As a result of the increasing availability of PCR and DNA sequencing facilities, 16S rRNA has become mandatory for identification and classification of novel bacterial strains (Snel et al. 1999). The part of the DNA now most commonly used for taxonomic purposes for bacteria is the 16S rRNA gene (Garrity and Holt 2001, Harmsen and Karch 2004, Kolbert and Persing 1999, Palys et al. 1997, Tortoli 2003). The 16S rRNA gene is also designated 16S rDNA, and the terms have been used interchangeably, recent ASM policy is that "16S rRNA gene" be used in general (Clarridge 2004).

In the 1960s, Dubnau and workers (Dubnau et al. 1965) noted conservation in the 16S rRNA gene sequence in *Bacillus* spp. Wide-spread use of this gene for bacterial identification and taxonomy followed a pioneering work by Woese (1987) who defined important properties of 16S rRNA gene which can be used as a molecular chronometer. The degree of conservation is assumed to result from the importance of the 16S rRNA as a critical component of cell function (Pfister et al. 2003). Use of 16S rRNA gene for bacterial identification of close relationships at the genus and species level, is very popular in clinical microbiology (Garrity and Holt 2001). The 16S rRNA gene is about 1,550 bp long and is composed of both variable and conserved regions. The 16S rRNA gene is large enough, with sufficient interspecific polymorphisms to provide distinguishing and statistically valid measurements. Universal primers are usually chosen as complementary to the conserved regions at the beginning of the gene and at either the 540 bp region or at the end of the whole sequence (about the 1,550 bp region), and the sequence of the variable region in between is used for the comparative taxonomy (Relman 1993). Although 500 and 1,500 bp is common lengths to sequence and compare, sequences in databases can be of various lengths. The 16S rRNA gene sequence has been determined for a large number of strains. GenBank has over 20 million deposited sequences, of which over 90,000 are of 16S rRNA gene. Importantly, the 16S rRNA gene is universal in bacteria and so relationships can be drawn among all bacteria (Woese et al. 1985, Woese 1987). In general, the comparison of the 16S rRNA gene sequences allows differentiation between organisms at the genus level across all major phyla of bacteria, in addition to classifying strains at multiple levels, including species and subspecies. The occasional exceptions to the usefulness of 16S rRNA gene sequencing usually relate to more than one well known species having the same or

very similar sequences. It is also important to consider whether it is necessary to sequence the whole 1,500 bp length or whether the commonly reported shorter sequences can provide comparable information. Sometimes sequencing the entire 1,500 bp region is necessary to distinguish between particular taxa or strains (Sacchi et al. 2002, Sacchi et al. 2002). Sequencing of the entire 1,500 bp sequence is also desirable and usually required when describing a new species.

2.10 Screening of *Bt* toxic gene by PCR

The PCR is a molecular tool widely used to characterize the insecticidal bacterium *Bt* strain collections (Gleave et al. 1993, Bravo et al. 1998, Ferrandis et al. 1999). *Bt* produces insecticidal toxin proteins during sporulation (Höfte and Whiteley 1989) encoded by different *cry* genes. There is another toxin protein, cytolytic (cyt), which basically enhance the effectiveness of cry toxins. Insecticidal activity of *Bt* depends on these cry toxins which in turn varies from insect to insect (Apaydin et al. 2005, Ghelardi et al. 2007, Konecka et al. 2007). The mammalian gut lacks the specific toxin receptors, otherwise present in insect gut thus making the former safe from these toxins (Crickmore 2006). Identification of *cry* gene content by PCR is the most effective technique in screening large native collection for predicting insecticidal activities of individual strains (Ben-Dov et al. 1997, Porcar and Juárez-Pérez 2003). The PCR based identification of *Bt* genes was first developed by Carozzi et al. (1991) and they have designed primers for *cry* 1A, *cry* 3A and *cry* 4A genes for identification of Lepidoptera, Coleoptera and Diptera active strains, respectively. Moreover, *cry*1, *cry*2 and *cry*9 genes were found to be active against Lepidopteran insects (Zhong et al. 2000). PCR has been shown to be a fast and accurate method for identification of the unknown *cry* genes with new insecticidal activity (Juárez-Pérez et al. 1997). In the

recent times, PCR has been used extensively to determine the sequence of *cry* gene from *Bt* strains. As yet, more than 100 pairs of specific and different primers have been designed to identify the *cry* genes subsets (Nariman 2007). More than 300 crystal proteins have been isolated and classified into 53 various groups on the basis of similarity in their sequences (Tohidi 2013).

The *cry* genes can be located in plasmid DNA or genomic DNA. When present in plasmid, they are associated with plasmid of large molecular mass (González 1981). Each *Bt* strain can carry one or more crystal toxin genes, therefore, strains of the organism may synthesize one or more crystal protein (Thomas et al. 2001). Cry proteins have been used as bio pesticide sprays on a significant scale for more than 50 years, and their safety has been demonstrated (Schnepf et al. 1998).

3. OBJECTIVES OF THE STUDY

a. Screening of sporadically occurring lepidopteran tea pests (mainly caterpillars) for bacterial pathogens. The lepidopteran species will include i. *Orgyia postica,* ii. *Andraca bipunctata* and iii. *Arctornis submarginata*.

b. Isolation and preliminary characterization of the bacterial entomopathogen based on polyphasic, biochemical and molecular techniques.

c. Bioassay (LC_{50} study) of the isolated strains of bacteria on the concerned tea pests to find their efficacy as biocontrol agent.

d. Cross- infectivity testing of the bacterial strain to beneficial insect (silkworm).

4. MATERIALS AND METHODS

4.1 Periodic survey of tea plantations:

For collection of sporadic pest specimens (lepidopteran), different tea plantations of Darjeeling foothills as well as its adjoining plains, Terai and the Dooars regions were extensively surveyed. The tea gardens selected for survey were Sepoydhura T.E, Makaibari T.E, Ambotia T.E, Soureni T.E, Singel T.E, Goomtee T.E, Sungma T. E, Seeyok T.E and Singtam T. E of lower elevation of Darjeeling hills (Fig. 4.1; Fig. 4.4A), MatigaraT.E., Dagapur T.E., Nischintapur T.E., Mohorgong T. E., New Chamta T. E., Simulbari T.E., Panighata T.E., Trihana T. E., Kamalpur T.E., Atal T.E., Naxalbari T. E., Gungaram T.E., Gayaganga T. E. and Kamla T.E. from the Foothills or Terai region (Fig. 4.2; Fig. 4.4B), Bagracote T.E., Chalsa T.E., Samsing T.E., Nagrakata T.E., Grassmore T.E., Binnaguri T.E., Hantapara T.E., Madhu T.E., Kalchini T.E., Chuapara T.E., Bhatkhawa T.E., Kumargram T.E. and Sankosh T.E. from the Plains or Dooars region (Fig.4.3; Fig. 4.4C)

4.2 Collection of dead and moribund larvae:

Moribund and dead pest larvae were collected from natural populations occurring in tea gardens and those dying in laboratory reared population. After proper surface sterilization, dead larvae (from tea garden as well as laboratory reared) were stored in double distilled water at -20°C for extraction and study of pathogenic bacteria in future.

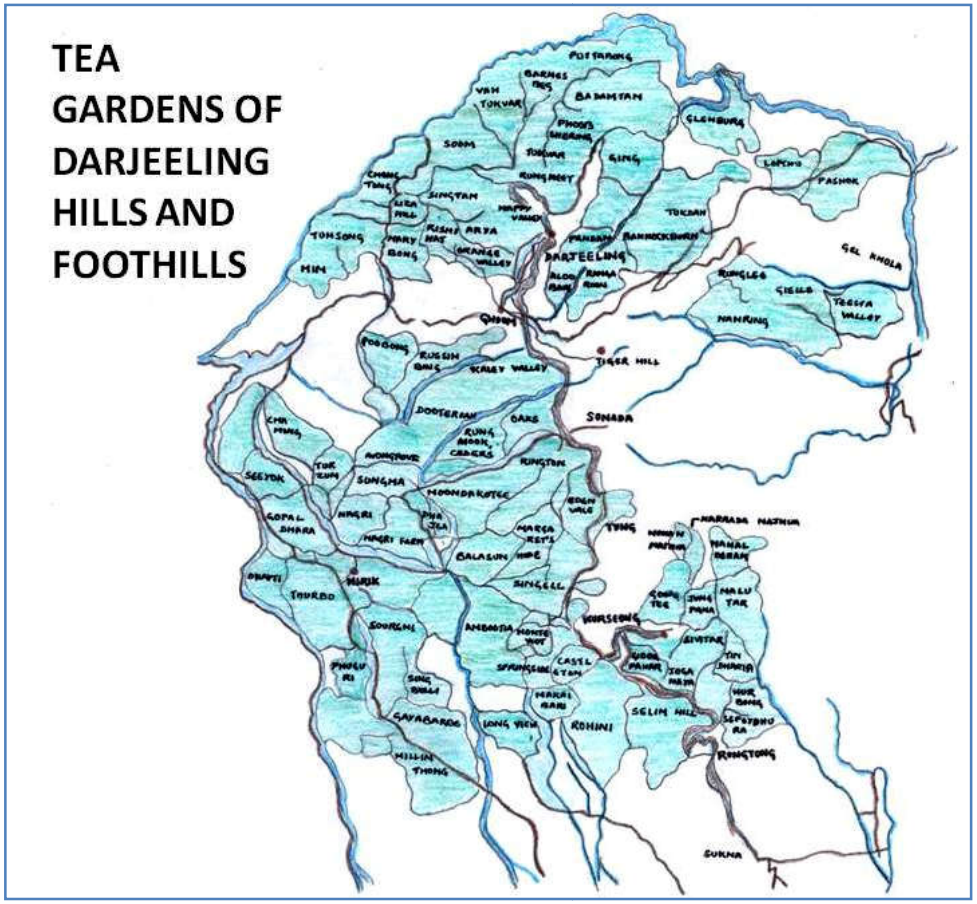


Fig. 4.1: Map showing tea gardens of Darjeeling hills and foothills.

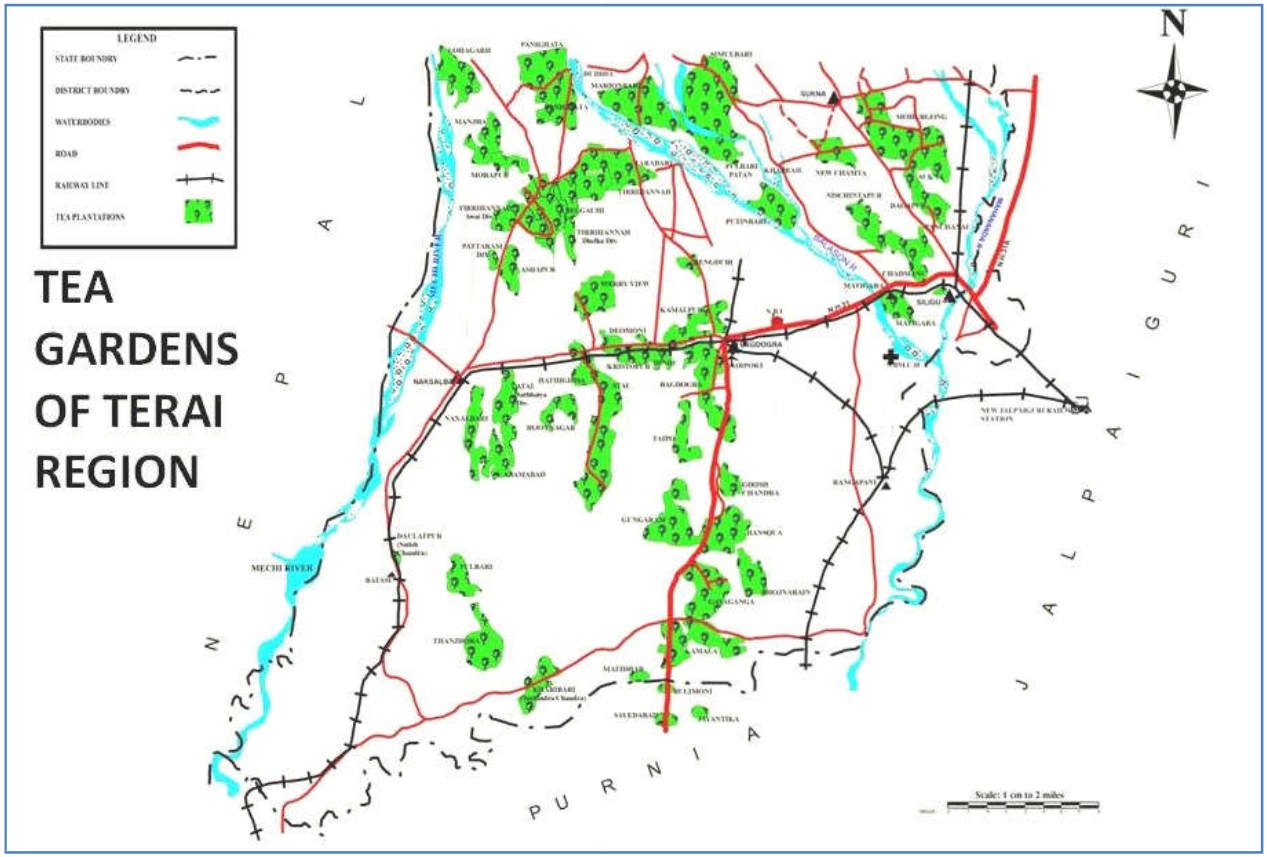


Fig. 4.2: Map showing tea gardens of the Terai region of Darjeeling district.

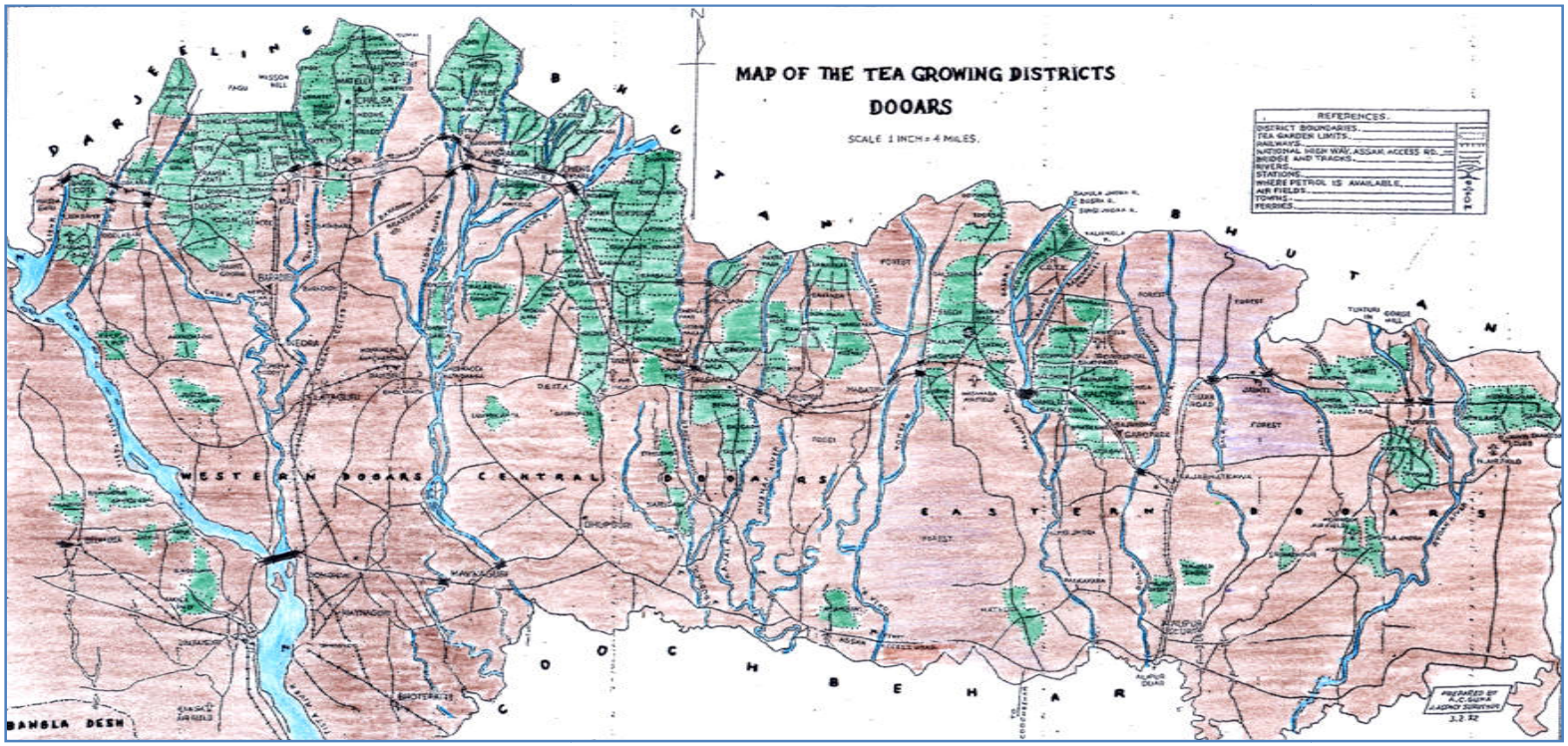


Fig. 4.3: Map showing the tea gardens of the Dooars region of Jalpaiguri and Alipurduar districts.

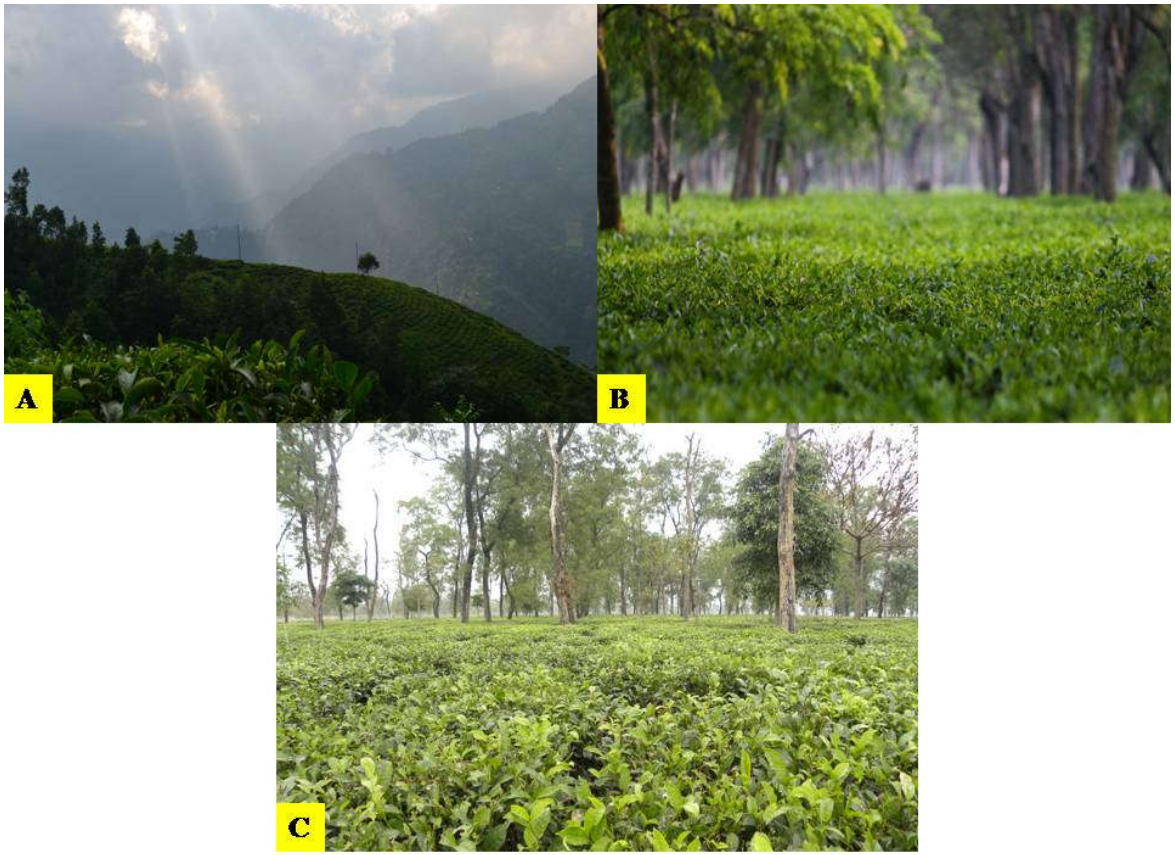


Fig. 4.4: Tea gardens of A) Darjeeling hill region (Soureni T.E), B) Terai region (Kamalpur T.E) and C) Dooars region (Kalchini T.E).

4.3 Rearing of insect

Larvae and moth of lepidopteran pests collected from different tea plantations were reared in laboratory in aseptic conditions for one generation. Fresh tea twigs/leaves of Tocklai vegetative clone (TV-25) from the experimental tea garden organically maintained at the Campus University of North Bengal was supplied as food.

4.4 Isolation of entomopathogenic bacteria:

For isolation of bacteria, dead larvae were dipped in 70% alcohol for two seconds for surface sterilization, washed three times with autoclaved double distilled water and stored at -20°C following Lacey and Brooks (1997). The stored dead larvae were thoroughly macerated by glass homogenizer and the crude homogenate was sieved through fine sterilized cheese cloth. The filtrate was centrifuged at 3000 rpm for 30 minutes. The supernatant was discarded and from the precipitate containing bacterial suspension, serial dilutions (10^{-1} to 10^{-7}) were made in sterile double distilled water. 100 µl of different dilutions were plated on Nutrient agar medium (0.5% Peptone, 0.3% beef extract/yeast extract, 1.5% agar, 0.5% NaCl and Distilled water, pH 7.4 at 25°C) by 'streak-plate inoculation method' incubated at 30°C in bacteriological incubator. Three replications for each dilution were taken for plating. After 24 hours, flat colonies of chalky white round smooth or fried egg appearance were picked up from mixed culture and again plated on to T3 medium (Tryptone 0.3%, Tryptose 0.2%, Yeast extract 1.5%, MnCl .0005%, 0.05 M Sodium phosphate and Distilled water, pH 6.8) and incubated at 30°C until lysis. All the selected colonies were purified using single colony isolation and spotted on to a master plate. The infectivity was determined following Koch's postulates by infecting healthy first instar larvae

with this isolated bacterium. After proving the Koch's postulates (Koch 1876, Black 1996, Fredericks and Relman 1996, Martin et al. 2008) the bacterial viability was checked by inoculating in new agar media.

4.5 Screening and identification of entomopathogenic bacteria from tea pests

4.5.1. Morphological characteristics:

4.5.1.1 Microscopic study

- **Gram's staining**

Overnight grown culture was smeared on a slide, air dried and then heat fixed. The cells were stained with crystal violet stain for about one minute and then washed with distilled water for a few seconds using a wash bottle. The slides were then trated with Gram's iodine for about one minute followed by 95% alcohol for 10 seconds and washed with distilled water. The slides were counter stained with safranin for one minute and washed in gentle stream of water. The slides were air dried and observed under oil immersion objective (100X) of a phase contrast microscope (Olympus- CX31). Those bacterial cell appearing as purple violet were referred to as Gram-positive and those appearing pink were described as Gram-negative (Fig. 4.5A) (Murray et al., 1999).

- **Crystal protein staining**

The isolates were grown in LB broth at 37°C under constant shaking. Overgrown culture (i.e. after 3-4 days) was taken, smeared on a slide, air dried and then heat fixed. The slides were stained with Coomassie blue for about 3 minutes and then washed in a gentle stream of distilled water. The slides were air dried and then

observed under oil immersion objective lense of a phase contrast microscope (Fig. 4.5B) (Sharif and Alaeddinoĝlu 1988).

- **Endospore staining**

Over grown culture was taken, smeared uniformly on a glass slide, air dried and then heat fixed. The slides were covered with a piece of blotting paper, flooded with a few drops of Malachite green (Sigma) and then steamed in water bath for 10 minutes. The blotting paper was removed and the slides were washed with a gentle stream of distilled water. The slides were counter stained with safranin for 30 seconds and washed with distilled water. The slides were air dried and observed under oil immersion objective for presence of green endospores. Once stained the endospores do not readily decolorize and appear green within red cells (Fig. 4.5C) (Hussey and Zayaitz 2007).

4.5.1.2 Motility test

Motility of the bacteria was observed by Hanging drop method of Cappuccino and Sherman (1996). A clean coverslip containing a drop of liquid culture was covered by a depression slide (Petroleum jelly was applied as ring on the depression slide). The slide was turned quickly so that the drop continues to adhere to the inner surface of the coverslip. The slides were observed under 40X of phase contrast microscope for motility assessment. Commercially available *Bacillus thuringiensis kurstaki* (*Btk*) was used as control for comparative study which is used as biopesticides in many organic gardens.

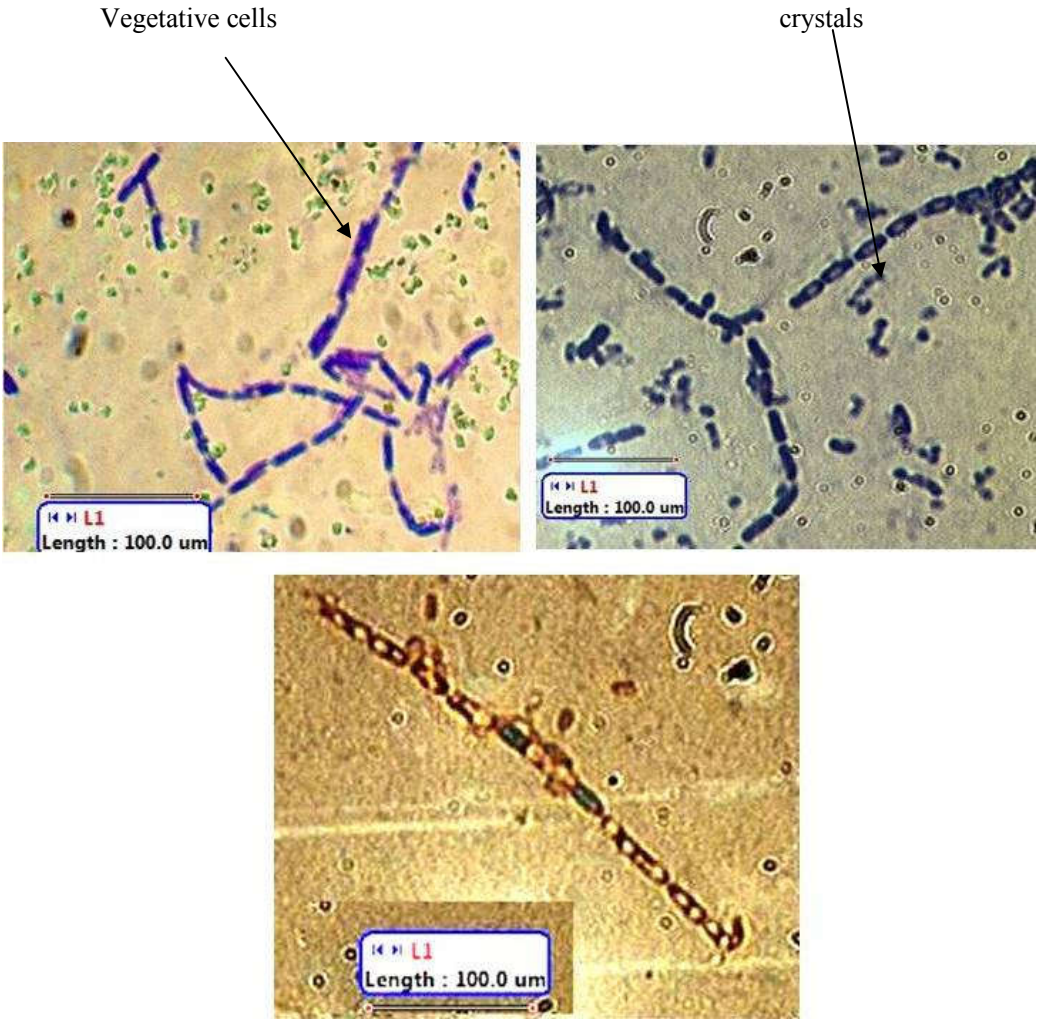


Fig. 4.5A: Gram's staining; B. Crystal protein staining C. Endospore staining of bacteria.

4.5.2 Biochemical characteristics:

Using API kit

Biochemical analyses like Indole, Voges-Proskour, Methyl red, Citrate utilization, Esculin hydrolysis, Lysine Decarboxylase, Ornithin Decarboxylase, H_2S production, Nitrate reduction, fermentation of different carbohydrates, Urease tests were performed using Biochemical testing kit (KB003, Himedia) with *Btk* as reference. This kit consists of medium for 24 biochemical tests and separate disc for oxidase test. 1-3 well isolated colonies were picked and a homogenous suspension was prepared in 2-3 ml distilled water.The density of the suspension was measured as 0.1 OD at 630 nm 200 µl of this suspension was transferred into each well of the test systems. To prevent any contact with air, the wells were filled up with mineral oil. Then the panels were incubated for 18-24 hrs at 37°C (Alsina and Blanch 1994). Oxidase test was done by picking up a single colony and rubbing it on the oxidase disc. Colour change was observed within 60 seconds. The biochemical tests and the results are summarized in the Table 4.1.

In addition to the above tests, the isolates were also characterized using three different biochemical tests for identification of entomopathogenic *Bacillus* sp. following the methods of Garrity (2001) and Aneja (2003) with sight modifications.

Table 4.1: Details of biochemical tests used for identifying bacterial strains.

Strip I	Result Interpretation chart					
No.	**Test**	**Reagents to be added after incubation**	**Principle**	**Original colour of the medium**	**Positive reaction**	**Negative reaction**
1	ONPG	—	Detects β – galactosidase activity	Colourless	Yellow	Colourless
2	Lysine utilization	—	Detects Lysine decarboxylation	Olive green to Light Purple	Purple / Dark Purple	Yellow
3	Ornithine utilization	—	Detects Ornithine decarboxylation	Olive green to Light Purple	Purple / Dark Purple	Yellow
4	Urease	—	Detects Urease activity	Orangish yellow	Pink	Orangish yellow
5	Phenylalanine Deamination	2-3 drops of TDA reagent	Detects Phenylalanine deamination activity	Colourless	Green	Colourless
6	Nitrate reduction	1-2 drops of sulphanilic acid and 1-2 drops of N, N-Dimethyl-1-Napthylamine	Detects Nitrate reduction	Colourless	Pinkish Red	Colourless
7	H_2S production	—	Detects H_2S production	Orangish yellow	Black	Orangish yellow
8	Citrate utilization	—	Detects capability of organism to utilize citrate as a sole carbon source	Green	Blue	Green
9	Voges Proskauer's	1-2 drops of Baritt reagent A and 1-2 drops of Baritt reagent B	Detects acetoin production	Colourless / Light Yellow	Pinkish red	Colourless/ slight copper
10	Methyl red	1-2 drops of Methyl red reagent	Detects acid production	Colourless	Red	Yellowish- orange
11	Indole	1-2 drops of Kovac's red reagent	Detects deamination of tryptophan	Colourless	Pinkish Red	Colourless
12	Malonate utilization	—	Detects capability of organism to utilize sodium malonate as a sole carbon source	Light green	Blue	Light green

Strip II	Result Interpretation chart				
No.	**Test**	**Principle**	**Original colour of the medium**	**Positive reaction**	**Negative reaction**
13	Esculin hydrolysis	Esculin hydrolysis	Cream	Black	Cream
14	Arabinose	Arabinose utilization	Pinkish Red / Red	Yellow	Red / Pink
15	Xylose	Xylose utilization	Pinkish Red / Red	Yellow	Red / Pink
16	Adonitol	Adonitol utilization	Pinkish Red / Red	Yellow	Red / Pink
17	Rhamnose	Rhamnose utilization	Pinkish Red / Red	Yellow	Red / Pink
18	Cellobiose	Cellobiose utilization	Pinkish Red / Red	Yellow	Red / Pink
19	Melibiose	Melibiose utilization	Pinkish Red / Red	Yellow	Red / Pink
20	Saccharose	Saccharose utilization	Pinkish Red / Red	Yellow	Red / Pink
21	Raffinose	Raffinose utilization	Pinkish Red / Red	Yellow	Red / Pink
22	Trehalose	Trehalose utilization	Pinkish Red / Red	Yellow	Red / Pink
23	Glucose	Glucose utilization	Pinkish Red / Red	Yellow	Red / Pink
24	Lactose	Lactose utilization	Pinkish Red / Red	Yellow	Red / Pink
25	Oxidase	Done on Oxidase disc separately. Detects cytochrome oxidase production.	Colourless	Deep purple within 10 seconds	White/ Purple after 60 seconds

- **Starch hydrolysis**

Starch hydrolysis test was carried out following the methods of Cowan and Steel (2004). Bacterial cultures were streaked on sterile Starch agar medium plate. The plates were incubated for 72 h at 30ºC. Later, the surfaces of the plates were flooded with iodine solution. The plates were examined for the starch hydrolysis around the line of the growth of organism. *E. Coli* inoculated starch agar medium plate was maintained as control (Fig. 4.6).

- **Casein hydrolysis**

Casein hydrolysis test was performed according to the method described by Cowan and Steel (2004). Bacterial cultures were streaked on sterile skim milk agar medium plate. The plates were incubated for 48 h at 30ºC. The plates were examined for clear area around the line of growth. *E.coli* inoculated skimmed milk agar medium plate was maintained as control (Fig. 4.7).

- **Catalase test**

Catalase test was performed following the method of Thiery and Frachon (1997). Nutrient agar slants were inoculated with the bacterial cultures. The cultures were incubated at 30ºC for 24 h. The tube was held at an angle and 3-4 drops of Hydrogen peroxide was dropped to flow over the growth of each slant. An uninoculated tube was kept for control. The culture was observed for appearance or absence of effervescence (Fig. 4.8).

4.5.3 Anaerobic growth

Anaerobic agar medium was distributed into culture tubes up to 7.5 cm and sterilized by autoclaving at 121^{o}C temperature and 15 pounds per square inch pressure for 20 minutes. The tubes were inoculated with a small (outside diameter 1.5 mm) loopful of

2 days old culture on nutrient agar medium by stabbing up to the bottom of the column. The tubes were incubated at 30^{o}C for 7 days and observed for growth along the length of the stab (anaerobic). An uninoculated tube was kept for control for the test (Claus and Berkeley 1986) (Fig. 4.9).

4.5.4 Similarity Matrix

The pair wise similarity based on matching biochemical result between pairs of different *Bt* strains was evaluated using the Dice Coefficient (SD) formula (Dice 1945).

SD =2a/ (n1+n2)

Where: a = the number of matching biochemical test result common between a pair of profiles, n1 and n2 = the total number of biochemical tests, respectively, in the first and second profiles.

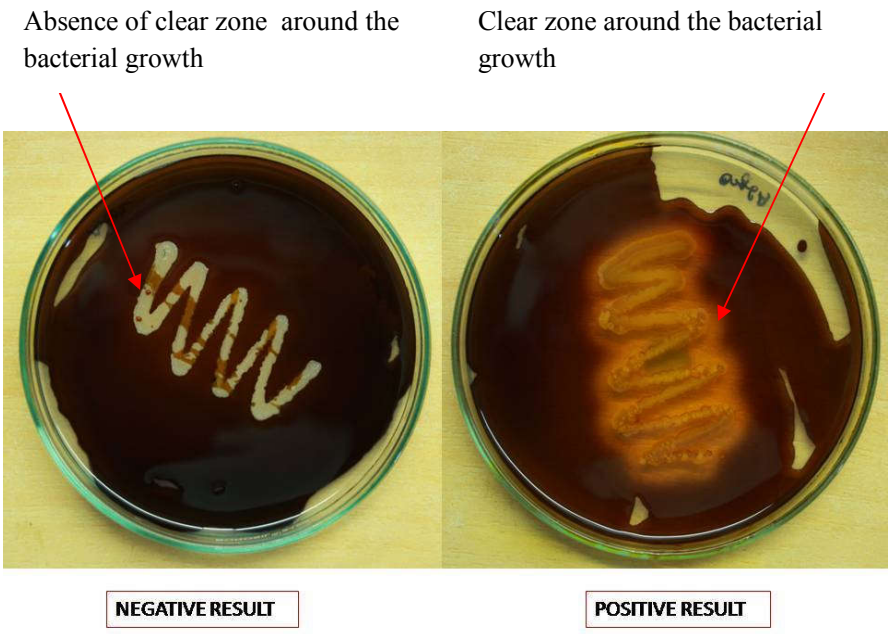


Fig. 4.6: Plates showing negative and positive starch hydrolysis result.

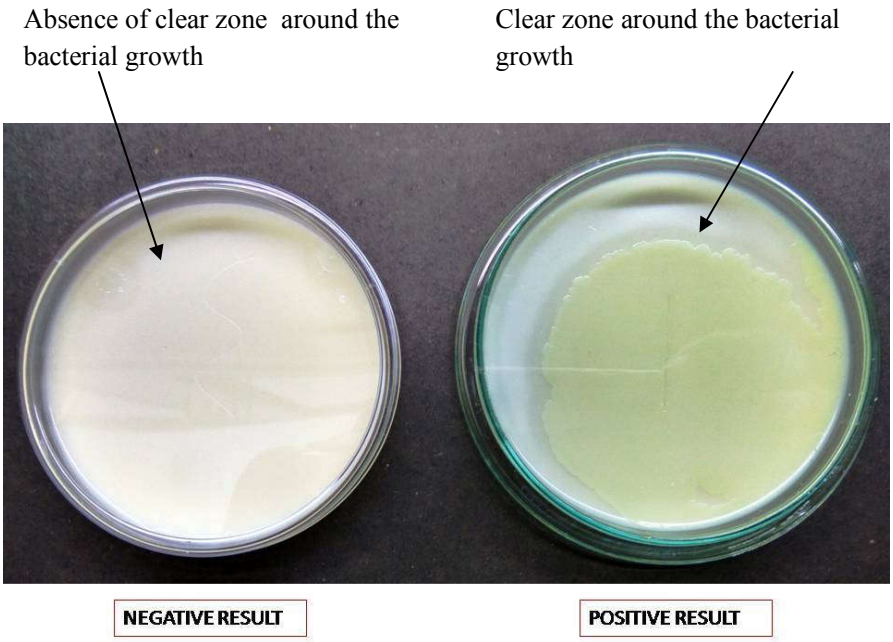


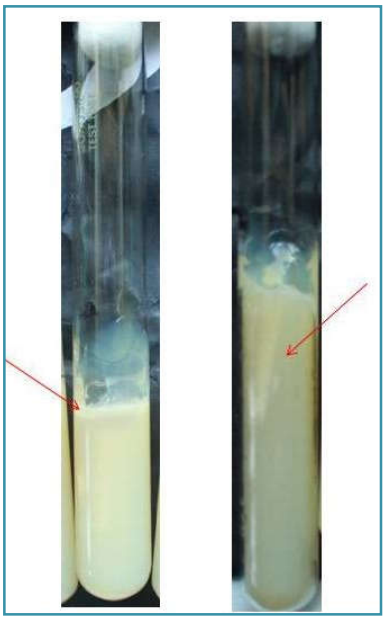
Fig. 4.7: Plates showing negative and positive casein hydrolysis result.

Absence of effervescence

Presence of effervescence

Fig. 4.8: Slants showing positive catalase test.

No growth along the length of the stab



Growth along the length of the stab

Fig. 4.9: Slants showing positive anaerobic growth of bacterium.

4.5.5 Physiological characteristics

4.5.5.1 Quantitative analysis of bacteria from larvae

Dead or moribund larvae were surface sterilized with 70% alcohol. The suspension obtained from each putrefied larvae were mixed separately in 5ml of sterilized phosphate buffer solution (PBS, pH 7.4) and filtered twice through two layers of cheese cloth to remove debris (Poinar and Thomas 1978). The suspension was diluted to 10^{-8} (Christine and Ted 1992). Each suspension was plated on nutrient agar and incubated at 30^{o}C for 24 hours. After incubation, the total number of bacteria in larvae was determined by counting the number of colonies on the plates, which were inoculated with diluted bacterial suspension.

4.5.5.2 Doubling time or Generation time

In this procedure, growth of the bacterial strain was determined by turbidimetric method of Cappuccino and Sherman (1996). The O.D. was measured at 540 nm every 30 minutes interval using a UV-Vis spectrophotometer (Rayleigh UV-2601). Finally log of O.D. and time interval were plotted on a graph paper (Fig. 4.10) and from this the generation time (doubling time) was determined using the graphical method. *Btk* was used as control for comparison. The generation time (GT) was calculated as follows:-

GT= t (OD at 0.8)- t (OD at 0.4)

4.5.5.3 Determination of colony forming unit/ ml

The number of organisms per ml of original culture was calculated by multiplying the number of colonies counted by the dilution factor (Cappuccino and Sherman 1996).

Number of cells per ml= number of colonies X dilution factor

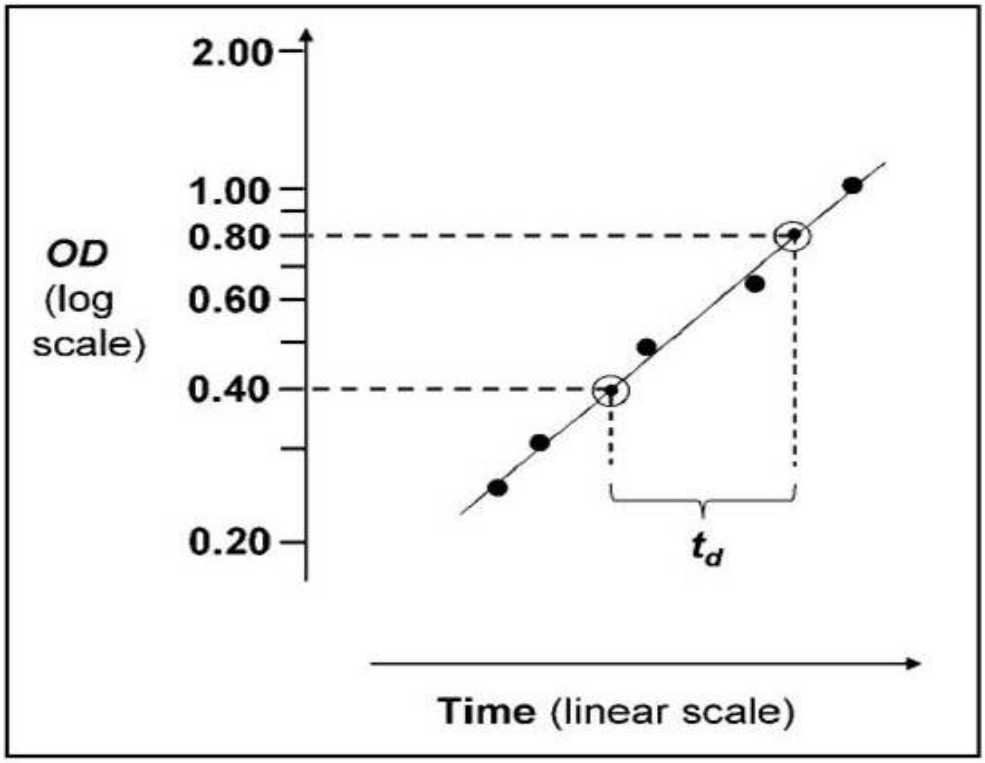


Fig. 4.10: Graph showing the generation time calculation.

4.5.6 SDS-PAGE analysis

4.5.6.1 Crystal protein of the bactera

The crystal protein was harvested by the method of Patel et al. (2009). Bacterial strains were grown in Luria Bertani medium at 37°C without shaking. It was grown up to the phase of sporulation. Sporulated culture of each isolate was pelleted down at 8000 rpm for 3 min and re-suspended in 50 µl sterile distilled water. 5 µl of 1 (N) NaOH was added after vortexing, and incubated for 5 min. 15 µl of Laemmli's buffer [4% SDS, 20% Glycerol, 10% 2-Mercaptoethanol, 0.004% Bromphenol Blue, 0.125 (M) Tris-HCl pH 6.8] was added and boiled for 2 min. The mixture was centrifuged at 8000 rpm for 3 min 20 µl sample was loaded per well of SDS-PAGE (10% Polyacrylamide gel, 100:1 Acrylamide/ Bisacrylamide ratio) as described by Laemmli (1970) and Quesada-Moraga et al. (2004). Gels were stained with staining solution (50% (v/v) ethanol, 10% (v/v) acetic acid and 0.1% (w/v) Coomassie brilliant blue R-250) for 40 min, and destained in a solution containing 6.75% (v/v) glacial acetic acid and 9.45% (v/v) ethanol. The bands were analysed using gel documentation system (Spectroline TVD 1000R/F).

4.5.6.2 Quantitative and qualitative analysis of whole cellular protein of the bacteria

The bacterium was cultured on Luria-Bertani (LB) agar for 24 hours at 37°C and the protein was extracted using 1% lysozyme solution and lysis buffer comprising 4% SDS, 20% glycerol, 2% 2-marcaptaethanol, 0.125 (M) Tris-HCL pH 6.8 and MiliQ water (Costas 1992). The extracted protein of each strain was divided into two parts. The first part was used for the quantitative protein analysis (total protein estimation) using UV- Visual spectrophotometer (Rayleigh UV-2601). The second part was used for SDS-PAGE analysis.

- **Quantitative analysis by Spectrophotometer:**

The total protein in each tested strains was measured spectrophotometrically following the method of Haggag and Yousef (2010). About 0.02 ml of protein solution was added to 1ml of reagent (1) [32 mM potassium sodium tartrate; 200 mM sodium hydroxide; 30 mM potassium iodide, and 12 mM copper sulphate]. For preparation of standard sample, 0.02 ml of standard Bovine serum albumin (BSA) was mixed with 1 ml of reagent (1). The samples were mixed well by vortex and left for 30 min at room temperature. The absorbance of sample (As) and that of standard (A std.) were measured against reagent blank at 545 nm. The protein concentration was calculated according to the standard equation.

- **SDS-PAGE analysis of whole cellular protein of bacteria**

The second part of cellular protein was used for qualitative analysis using SDS-PAGE. Gels were stained with 50% ethanol, 10% acetic acid and 0.1% (w/v) Coomassie brilliant blue R-250 for 40 min and destained in a solution containing 6.75% (v/v) glacial acetic acid and 9.45% (v/v) ethanol. The bands were analysed using gel documentation system (Spectroline TVD 1000R/F).

- **Plasmids of the isolates**

were purified by alkaline lysis method of Ehrt and Schnappinger (2003) with some modifications. 2 ml of overnight culture grown in LB broth at 30ºC was pelleted and re-suspended in 100µl of TE buffer [(40 mM Tris-HCL, 2 mM EDTA (pH 7.9)]. To it 200µl of lysis solution [3% SDS, 15% sucrose, 50 mM Tris-hydroxide, (pH 12.5)] was added and incubated for 30 min at 60ºC. Then 2µl of proteinase K was added, mixed gently and incubated for another 90 min at 37ºC. 1 ml of phenol (Tris saturated) was added to each tube vortexed and centrifuged (SIGMA 3K30) at 8000

rpm for 2 min. The upper aqueous phase was collected in a separate tube and 1ml of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the mixture and the tubes were inverted carefully several times and centrifuged at 8000 rpm for 2 min. The upper aqueous phase was collected in a separate tube and equal volume of chloroform was added to each tube and inverted several times and centrifuged at 8000 rpm for 2 min. The upper aqueous phase was collected in fresh tubes and $1/10^{th}$ the volume of supernatant, 3M sodium acetate (pH 5.2) was added to each tube. Then 0.8 volume of isopropanol was added to the supernatant mixed throughly and kept at RT for 1hr. The samples were then centrifuged at 13000 rpm for 15 min at 4°C and the supernatant harbouring plasmid DNA was separated carefully. The supernatant was discarded and 500 µl cold 70% ethanol was added to each tubes. The mixture was centrifuged at 10000 rpm for 5 min at 4ºC. The DNA was air dried and dissolved in 50 µl TE [(1 M Tris-HCl, 0.5 M EDTA (pH 8.0)] and sotred at 4ºC for future use. Plasmids of the isolates were then electrophoresed in 0.8% agarose gel prepared in 1X TAE [(40mM Tris, 20mM Acetate, 1mM EDTA (pH 8.6)] buffer containing 0.5 µg/ml of ethidium bromide. *Hind* III and *Eco R*I double digested λ DNA was used as the size marker. Gels were visualized on gel documentation system (Spectroline TVD 1000R/F) and photographed with transmitted UV light using Olympus camera.

4.6 Bioassay of bacterial isolates and determination of LC_{50} value and LT_{50} value

The efficacy of the entomopathogenic bacteria was determined by bioassay. Extraction of spore-crystal was done following the method of Shishir et al. (2012). Isolates with crystal bodies was cultured in 20ml of T_3 liquid medium and incubated for 7 days at 30 °C with regular shaking manually. Aliquots of 1.5 ml liquid culture

was then centrifuged at 5000 rpm for 15 min. Pellet (spore and crystal mixture) was washed twice with 1 ml sterile distilled water by centrifuging at 5000 rpm for 15 min. Next weight of pellet was taken and re-suspended in 1 ml of distilled water. The suspension was kept at 4°C for future use. Bioassay was done after the procedure of Dulmage et al. (1970). Different concentrations (100, 300, 500, 750 and 1000 μg/ml) of crude samples (spores and crystal) were used in the LC_{50} (median lethal concentration) and LT_{50} (median lethal time) bioassay by spreading these uniformly on tea leaves offered as food according to the procedure of Unnamalai and Vaithilingam (1995). For conducting bioassay ninety second instar larvae from laboratory culture were used for each bacterial concentration. The experiment was set up in 3 replicates each containing 30 caterpillars (Fig. 4.11). Sterile distilled water applied leaves were used as control. The mortality was observed at an interval of 24 hrs from the day of inoculation (1st day feeding up to 9th day). The mortality caused by each concentration was corrected for the control rate of mortality in each assay by using Abbott's formula (Abbott 1925). The corrected per cent mortality was calculated using Abbots formula, as follows:

$$\textbf{Corrected mortality (\%)} = \frac{X-Y}{X} \times 100$$

X = % live insects in control,; Y = % live insects in treatment.

Data were subjected to probit analysis (Finney and Tattersfield 1952) and median lethal concentration (LC_{50}) value was calculated from the regression equation.

Median lethal time (LT_{50}) value was also determined simultaneously following the method of Biever and Hostetter (1971):

$$\mathbf{LT_{50} = a + e\,(c-b)/D}$$

Where, a = the number of hours from the initiation of the test until the reading made just before the 50% value was recorded, b = the total number of larvae dead at the reading just before 50% value was recorded, c = 50% of the total number tested, D= the number of larvae dying in 24 hr period during which the 50% mortality was reached, and e = the number of hours between mortality counts.

4.6.1 Cross infectivity testing on mulberry silkworm

The cross infectivity of isolated entomopathogenic bacteria was tested by exposing beneficial Lepidopteran, like silkworm (*Bombyx mori*) separately. As the sericulture industry is running side by side with the tea industry in North Bengal so it is necessary to determine the toxicity/infectivity of the bacterial isolate to silk worms before trying the isolates in the field. For testing the infectivity in the laboratory condition multivoltine silkworms were taken (Fig. 4.12). Disease free layings (DFLs) were collected from West Bengal Sericulture Department situated at Matigara, Siliguri. Newly hatched neonates were fed with fresh and sterilized mulberry leaf collected from University of North Bengal campus and from West Bengal Sericulture Department, Matigara. The second instar larvae were taken for the cross infectivity test. Crude spore-crystal mixture (100, 300, 500, 750 and 1000 µg/ml) were prepared in distilled water. Ninety second instar caterpillars were taken for each treatment with varying concentrations of bacteria (Three replicates each containing 30 caterpillars). Similarly 90 caterpillars were taken for control experiment. Number of mortality was counted every 24 hours from the day of inoculation, (1^{st} day feeding up to 9^{th} day).

Fig. 4.11: Bioassay setup in laboratory.

Fig. 4.12: Silk worm (*Bombyx mori*) larvae feeding mulberry leaves.

4.7 Genomic DNA extraction for PCR analysis

The genomic DNA was isolated following the methods of (Sambrook et al. 1989, Juárez-Pérez et al. 1997, Sambrook and Russell 2001) with some modifications. 1.5 ml of overnight grown culture was centrifuged at 8000 rpm at room temperature for 5 min and the pellet was collected by discarding the supernatant. The pellet was resuspended in 567 µl TE (10mM Tris-HCl pH 8.0 and 1mM EDTA pH 8.0). 3µl Proteinase K (20 mg/ml) and 30µl 10% SDS were then added to the suspension. The mixture was incubated for 1 hr at 37ºC which turned the solution clear and viscous. To this 100 µl of 5 M NaCl was added and mixed well. Finally 80µl CTAB/NaCl solution was added and the mixture was incubated for the second time at 60ºC for 10 min. An equal volume (750µl) of Tris-saturated phenol: chloroform: isoamyl alcohol (25:24:1) was added and centrifuged at 10000 rpm for 15 min at room temperature. The upper aqueous phase was transferred to a new tube and equal volume (750µl) of chloroform: isoamyl alcohol (24:1) was added, mixed and centrifuged at 10000 rpm for 10 min. the upper aqueous phase was transferred to a new tube and 450µl of isopropanol was added and mixed properly until a clump of DNA is visible. The mixture was centrifuged at 10000 rpm for 5 min at 4ºC, the pellet was washed with 1 ml of 70% ethanol for 30 sec and centrifused at 10000 rpm for 5 min at 4ºC. Finally the pellet (DNA) was air dried and dissolved in 30-50 µl TE buffer and sotred at 4ºC for future use. Genomic DNA of the isolates were then electrophoresed in 1% agarose gel prepared in 1X TAE buffer containing 0.5 µg/ml of ethidium bromide. *Hind* III and *Eco R*I double digested λ DNA was used as the size marker. Gels were visualized on gel documentation system (Spectroline TVD 1000R/F) and photographed with transmitted UV light using Olympus camera.

4.7.1 Quantification of DNA

Amount of DNA was calculated by using the following formulae (Sambrook and Russell 2001).

1 O. D. at 260 nm = 50 µg of double stranded DNA/ ml

Amount of double stranded DNA = (X) x 50/2

Where, X = absorbance of the sample at 260 nm

Purity of the total DNA isolated was also tested by using spectrophotometer based on $^{A}280/260$ and also checking the integrity of the DNA by running in 1% agarose gel. The extracted DNA was used for amplification of specific sequence by Polymerase chain reaction (PCR).

4.7.2 PCR amplification for 16s rRNA sequence

PCR was performed with Universal 16S rRNA gene specific primer pair (Table 4.2). The PCR was set up in 0.2 ml PCR tubes. The components of the reaction mixture have been shown in the Table 4.3. The contents were mixed followed by a brief spin. PCR was carried out in a 96 well thermal cycler (Eppendorf) with the following cycling program: lead heat at 105°C, initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 1 min, primer annealing at 50 °C for 30 s, and primer extension 72 °C for 7 min. The product was stored at 4°C. The 16S rRNA sequence of the purified PCR product was determined using an Applied Biosystems model 3130 DNA sequencer and the ABI PRISM cycle sequencing kit. The sequence obtained were aligned using MEGA 5 and compared with available sequences from NCBI GeneBank using BLAST program (Altschul et al. 1997).

4.8 Identification of *cry* gene

Colony PCR was done using rapid cell lysate as the DNA samples after the method of Apaydin et al. (2005) and Valicente and Lana (2010). *Bacillus* strains were grown on Luria agar plate for 12h. A loopful of cells was transferred to the following ml of sterile distilled water and frozen at -20° C for 20 min. The mixture was transferred to boiling water for 10 min to lysate the cells. The resulting cell lysate was briefly spun for 10 sec at 10000 rpm. 2µl of this suspension was added as template to specific primers for *cry* genes (Table 4.4). The reaction mixture for PCR has been shown in the Table 4.5.

4.8.1 PCR amplification of *cry*1, *cry* 2 and *cry* 9 genes.

The PCR was carried out for the amplification of *cry* 1, *cry* 2 and *cry* 9 genes using the primer sets (Table 4.4) separately. All the ingredients (Table 4.5) were mixed followed by a brief spin. PCR was carried out in a 96 well thermal cycler (Eppendorf) with the following cycling program: lead heat at 105°C, initial denaturation at 94 °C for 2 min, 30 cycles of denaturation at 49 °C for 45s for *cry* 1, 54°C for *cry* 2 and 61°C for *cry* 9 and primer extension at 72 °C for 2 min, with a final extension at 72 °C for 10 min. The product was stored at 4°C.

4.8.2 Agarose gel electrophoresis

The PCR products were seperated in 1% agarose gel prepared in 1x TAE buffer. About 5µl of the PCR amplified products were mixed with 1µl of 6x loading dye (0.48% SDS,0.03% Xylene Cyanol, 0.03% Bromophenol Blue) and loaded into the wells of agarose gel containing 0.5 µg/ml ethidium bromide (Sambrook et al. 1989).

A 100 bp step up ladder was used as size marker. The electrophoresis was carried out with a constant voltage of 70 Volts in 1x TAE until the dye front migrated to nearly ¾th of the gel length. Gels were visualized on gel documentation system (Spectroline TVD 1000R/F) and photographed with transmitted UV light using Olympus camera.

4.9 Statistical analysis

Quantitative data were subjected to standard statistical analysis. Software packages such as SSP and Origin 4.0. MEGA5 were used for sequence alignment. For bioassay, cross infectivity, results were determined with the help of probit analysis. MS-EXCEL was used in all the experiments.

Table 4.2: Primer pair used for amplification of 16S rRNA gene.

Primer pair	Sequence (5' to 3')[a]	Size of amplicon (bp)	Annealing temperature (°C)	Reference
Universal 16S rRNA gene specific primer pair	AGA GTT TGA TCC TGG CTC AG ACG GCT ACC TTG TTA CGA CTT	1500	50	(Weisburg et al. 1991)

a The top primer is the sense primer and the bottom primer is the antisense primer.

Table 4.3: Reaction mixture for PCR for 16S rRNA gene.

Sl.	No Reaction mixture	
1	10 X *Taq* Buffer (2mM)	3.0µl
2	$MgCl_2$	1.5 µl
2	dNTPs mix (1mM)	2.0µl
3	Forward primer (-) 20 pmoles	1.0µl
4	Reverse primer, (+)20 pmoles	1.0µl
5	*Taq* DNA polymerase(1U/µl)	0.20µl
6	Template DNA (200 ng/µl)	2.0µl
7	Milli Q water	15.67µl
	Total	25.0µl

Table 4.4: Primers for *cry* 1, *cry* 2 and *cry* 9 genes.

Gene	**Sequence** (5' to 3')[a]	**Expected product size (bp)**	**Reference**
***cry* 1**	CATGATTCATGCGGCAGATAAAC TTGTGACACTTCTGCTTCCCATT	**277**	(Ben-Dov et al. 1997)
***cry* 2**	GTTATTCTTAATGCAGATGAATGGG CGGATAAAATAATCTGGGAAATAGT	**1500**	(Sauka et al. 2005)
***cry* 9**	CGGTGTTACTATTAGCGAGGGCGG GTTTGAGCCGCTTCACAGCAATCC	**354**	(Ben-Dov et al. 1997)

a The top primer is the sense primer and the bottom primer is the antisense primer.

Table 4.5: Reaction mixture for PCR for *cry* genes.

Sl.	No Reaction mixture	
1	10 X *Taq* Buffer (2mM)	2.0µl
2	$MgCl_2$	2.4 µl
2	dNTPs mix (1mM)	4.0µl
3	Forward primer (-) 20 pmoles	2.0µl
4	Reverse primer, (+)20 pmoles	2.0µl
5	*Taq* DNA polymerase(1U/µl)	0.5µl
6	Template DNA	5.0µl
7	Milli Q water	7.1µl
	Total	25.0µl

5. RESULTS

5.1. Isolation of bacteria from three sporadic tea pests

5.1.1. Characteristics and symptoms of three infected pest species

Since the gut is the initial organ affected in bacterial infections, the first signs of disease are related to feeding and assimilation. Loss of appetite, cessation of feeding, diarrhoea, gut paralysis and regurgitation are characteristic initial symptoms of bacterial infections. The bacteria infected larvae of *Arctornis submarginata*, *Andraca bipunctata* and *Orgyia postica* showed significant shrinkage of body due to cessation of eating and discolouration followed by blacking first around the gut region then the whole body and rapid decomposition. The healthy larvae and dead larvae due to bacterial infection are evident in the Fig. 5.1 A, B; Fig. 5.2 A, B and Fig. 5.3 A, B.

5.1.2. Quantitative estimation of bacteria

Total number of bacteria per larva was determined by counting the number of colonies on the plates, which were inoculated with diluted bacterial suspensions. The total number of bacteria recorded per advanced larva was $5.631X10^6 \pm 0.313$ for *A. submarginata*, $5.793X\ 10^6 \pm 0.240$ for *A. bipunctata* and $4.316X10^6 \pm 0.268$ for *O. postica*.

5.1.3. Isolation and screening of the entomopathogenic *Bacillus* strains from the cadaver of three sporadic tea pests

Naturally occurring entomopathogenic bacteria were isolated from the dead larvae (n=50) of *A. submarginata*, *A. bipunctata* and *O. postica* collected from different tea gardens of Darjeeling Foothills, Terai and the Dooars.

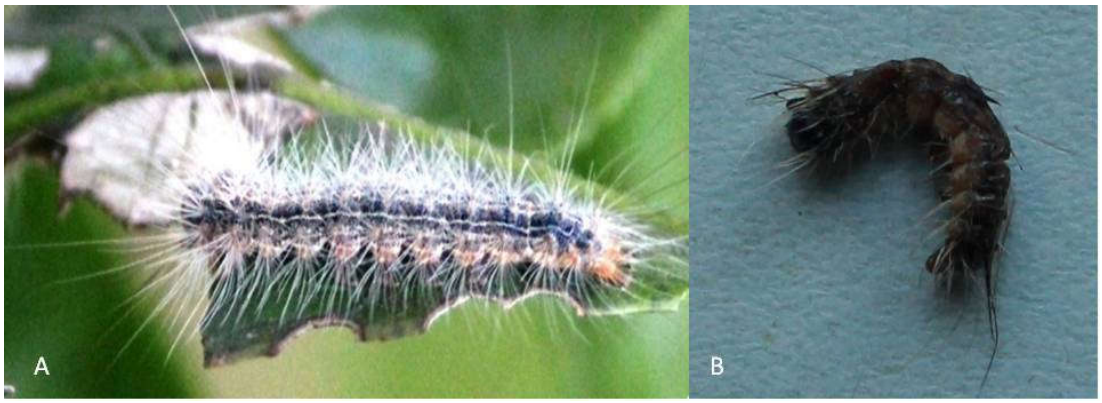


Fig. 5.1: *Arctornis submarginata* larva A) Healthy caterpillar; B) Bacterial infected cadaver.

Fig. 5.2: *Andraca bipunctata* larva A) Healthy caterpillar; B) Bacterial infected cadaver.

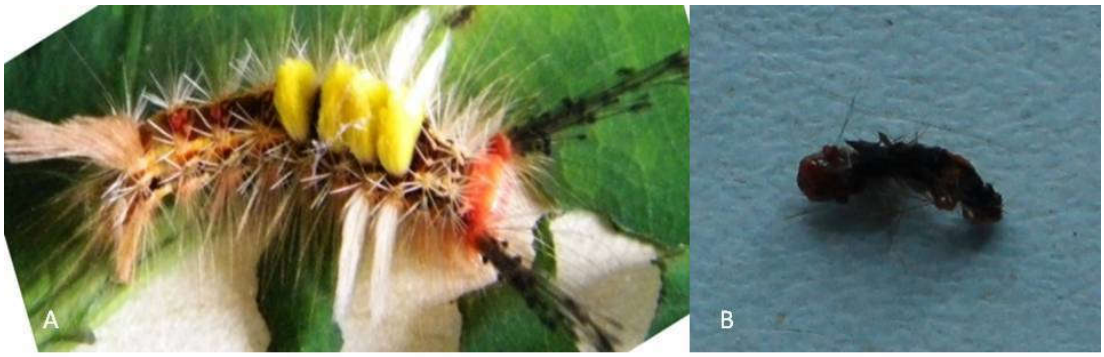


Fig. 5.3: *Orgyia postica* larva A) Healthy caterpillar; B) Bacterial infected cadaver.

I. *A. submarginata*: Out of 50 cadavers of *A. submarginata*, 21 seemed to harbour 23 *Bacillus* like colonies. Among these 23 *Bacillus* positive strains, seven (07) were picked according to the high propotion of bacteria in the cadavers. The same were tested for Koch's postulate. All the 07 bacterial strains were found to be positive for Koch's test. These were recorded as commonly occurring entomopathogens of *A. submarginata* caterpillar and were coded as Arc01-Arc07. Of these the most commonly occurring strains (Arc01, Arc02 and Arc03) were considered for detailed study (Fig. 5.4).

II. *A. bipunctata*: Among 50 cadavers of *A. bipunctata*, 25 cadavers showed 19 *Bacillus* like colonies (white and depressed with rough edge/ opaque and raised with smooth margins). Out of 19 *Bacillus* positive isolates, ten (10) were picked according to high proportion of bacteria in the cadavers. The same were tested for Koch's postulate. All the strains were positive to Koch's postulate and the most commonly occurring entomopathogens of *A. bipunctata* caterpillar were coded Ab01, Ab02, Ab03 and Ab04. They were considered for detailed study. The rest of the strains occurred in low proportion, as such only preliminary characterization has been done for them (Fig. 5.5).

III. *O. postica*: A total of 50 cadavers of *O. postica* were used for bacterial isolation, of which 22 cadavers had 18 *Bacillus* like colonies. Among these 18 bacterial isolates, six (06) were selected according to their occurrence in high proportion in the insect cadaver. They were tested for Koch's postulate and all fulfilled the test. The most commonly occurring entomopathogens against *O. postica* were coded Org 2A and Org 6A. These strains were considered for detailed study. The rest of the strains occurred in low proportion as such only preliminary characterization were done for them (Fig. 5.6).

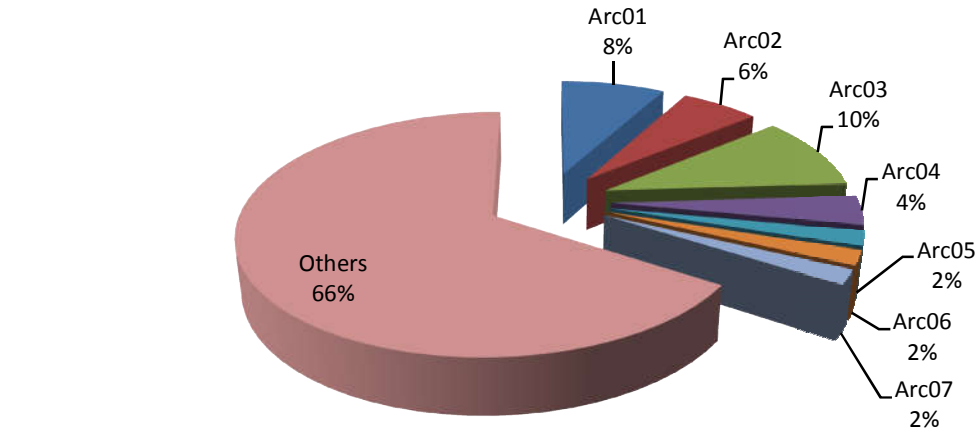


Fig. 5.4: Proportion of different *Bacillus* strains in *A. submarginata* cadavers.

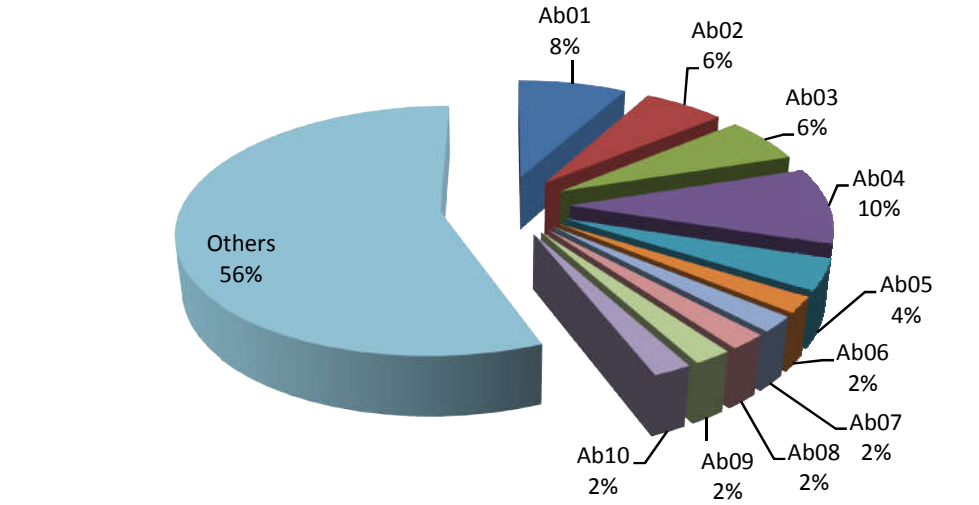


Fig. 5.5: Proportion of different *Bacillus* strains in *A. bipunctata* cadavers.

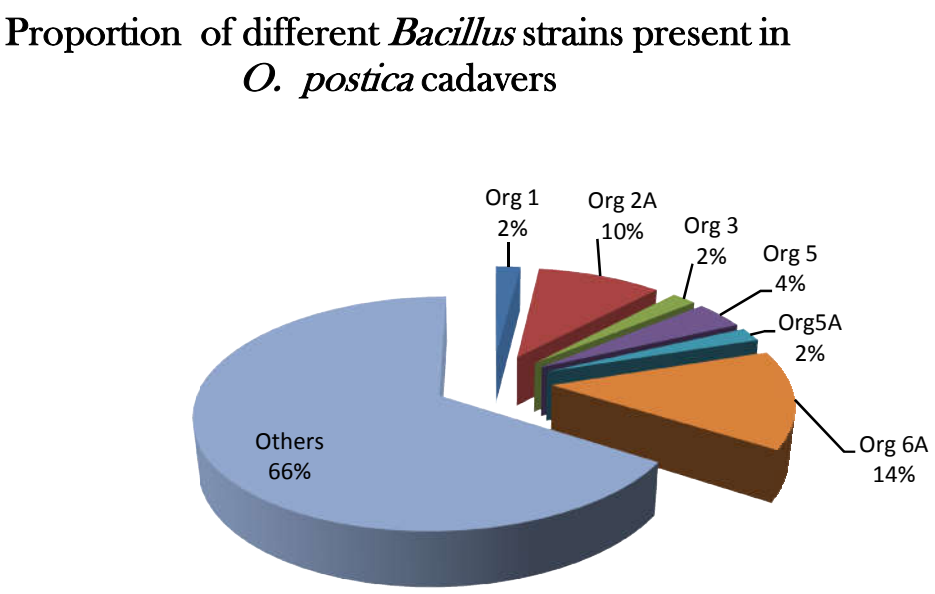


Fig. 5.6: Proportions of different *Bacillus* strains in *O. postica* cadavers.

After primary isolation, all the *Bacillus* strains from *A. submarginata* (07), *A. bipunctata* (10) and *O. postica* (06), respectively, were processed for further characterization (Table 5.1).

For convenience in addressing, describing and discussing these strains, in the forthcoming text of the thesis, designations along with coding of the strains have been done as mentioned in Table 5.2.

5.2. Characterization of the isolates

5.2.1. Physiological characteristics of the bacterial isolates

5.2.1.1. Determination of generation/ doubling time of the *Bacillus* strains

The generation/ doubling time of Arc01 isolated from *A. submarginata* was 84 min which was double of the generation time of *Btk* i.e. 42 min. The doubling time of Arc02and Arc03 was recorded as 20 min and 35 min, respectively. Further, it was found that the doubling time of other *Bacillus* isolates was i.e. Arc04, Arc05, Arc 06 and Arc07 were 72 min, 54 min, 51 min and 48 min, respectively (Table 5.3). All the isolates showed different generation time and Arc02 showed the least dobling time of 20 min followed by Arc03 which had a generation time of 35 min. Further, one way ANOVA of doubling times of different strains of *Bacillus* sp. isolated from

A. submarginata and reference strain *Btk* were found to be significantly different suggesting that all the isolates as well as *Btk* are different strains (Table 5.4).

Table 5.1: *Bacillus* sp. from dead larvae samples from tea gardens of Darjeeling foothills, Terai and Dooars.

Name of Tea Pests	Number of dead larvae samples	No. of cadavers harbouring *Bacillus* colonies	No. of *Bacillus* strains isolated	No. of Koch's postulate positive isolates
Arctornis submarginata	50	21	23	7
Andraca bipunctata	50	25	19	10
Orgyia postica	50	22	18	6

Table 5.2: Bacterial strains/isolates from *A. submarginata*, *A. bipunctata* and *O. postica* at a glance.

Name of Tea Pests	Code and Designations of the *Bacillus* strains isolated
Arctornis submarginata	Arc 01, Arc 02, Arc 03, Arc 04, Arc 05, Arc 06 and Arc 07
Andraca bipunctata	Ab 01, Ab 02, Ab 03, Ab 04, Ab05, Ab06, Ab07, Ab08, Ab09 and Ab10
Orgyia postica	Org 1, Org 2A, Org 3, Org 5, Org 5A and Org 6A

Table 5.3: Comparative account of doubling times of *Bacillus* sp. isolated from *A. submarginata* and reference strain *Btk.*

Name of Bacteria	**Doubling time (minutes)**
Btk	**42**
Arc 01	**84**
Arc 02	**20**
Arc 03	**35**
Arc04	**72**
Arc05	**54**
Arc06	**51**
Arc07	**48**

Table 5.4: ANOVA of generation/doubling times of different strains of *Bacillus* sp. isolated from *A. submarginata*.

ANOVA						
Source of Variation	SS§	df	MS¥	F	P-value	*F crit***
Between Groups	14227.5	7	203.25	203.25	1.64E-40	2.312741
Within Groups	32	32	1			
Total	14259.5	39				

§ Sum of squares

¥ Mean of squares

**Analysis showed that F> F crit (critical value). Therefore the null hypothesis was rejected and it was concluded that the variables (doubling time) are significantly different.

The generation/doubling time of four strains isolated from *A. bipunctata* were less than that of doubling time of *Btk.* Ab01, Ab02, Ab03 had doubling time of 36 min, 35 min and 33 min, respectively, whereas, Ab04 had the shortest doubling time of 24 min. Further, doubling time of rest of the six *Bacillus* strains of *A. bipunctata* were calculated as 62 min, 44 min, 53 min, 31 min, 38 min and 60 min for Ab05, Ab06, Ab07, Ab08, Ab09 and Ab10, respectively, (Table 5.5). All the isolates showed different generation time. Further, one way ANOVA of doubling times of different strains of *Bacillus* sp. isolated from *A. bipunctata* and reference strain *Btk* were found to be significantly different suggesting that all the isolates as well as *Btk* are different strains (Table 5.6).

The generation/doubling time of the isolates of *O. postica*, Org1, Org 2A, Org3, Org5, Org 5A and Org 6A were 69, 66, 74, 86, 82 and 30min respectively. Except Org 6A, all isolates took more time to double than *Btk*, which took 42 min to double (Table 5.7). One way ANOVA of doubling times of different strains of *Bacillus* sp. isolated from *O. postica* and reference strain *Btk* were found to be significantly different suggesting that all the isolates as well as *Btk* are different strains (Table 5.8).

5.2.1.2. Colony forming unit/ml of *Bacillus* strains

Microbial growth can be quantified by counting the total number of viable colonies formed by the inoculums of bacteria on the nutrient agar plate during one generation time. Assumption is that each cell in the aliquot can form one colony forming unit on the solid media. The cfu/ml was calculated to be $17.76x10^9$, $14.33x10^9$, $19.31x10^9$ for Arc01, Arc02 and Arc03, 8.15x109, $11.45x10^9$, $11.38x10^9$, $14.54x10^9$ for Ab01, Ab02, Ab03 and Ab04, while cfu/ml was found to be $10.54x10^9$ and $17.84x10^9$ for Org 2A and Org 6A, respectively. Reference strain *Btk* had the cfu/ml of $16.3x10^9$.

Table 5.5: Comparative account of doubling times of *Bacillus* sp. isolated from *A. bipunctata* and reference strain *Btk.*

Name of Bacteria	**Doubling time (minutes)**
Btk	**42**
Ab 01	**36**
Ab 02	**35**
Ab 03	**33**
Ab 04	**24**
Ab05	**62**
Ab06	**44**
Ab07	**53**
Ab08	**31**
Ab09	**38**
Ab10	**60**

Table 5.6: ANOVA of doubling times of different strains of *Bacillus* sp. isolated from *A. bipunctata*.

ANOVA

Source of Variation	SS§	df	MS¥	F	P-value	*F crit***
Between Groups	7372.727	10	737.27	737.27	1.5E-45	2.053901
Within Groups	44	44	1			
Total	7416.727	54				

§ Sum of squares

¥ Mean of squares

**Analysis showed that F> F crit (critical value). Therefore the null hypothesis was rejected and it was concluded that the variables (doubling time) are significantly different.

Table 5.7: Comparative account of doubling times of *Bacillus* sp. isolated from *O. postica* and reference strain *Btk.*

Name of Bacteria	**Doubling time (minutes)**
Btk	**42**
Org 2A	**66**
Org 6A	**30**
Org1	**69**
Org3	**82**
Org5	**74**
Org5A	**86**

Table 5.8: ANOVA of doubling times of different strains of *Bacillus* sp. isolated from *O. postica*.

ANOVA

Source of Variation	SS[§]	df	MS[¥]	F	P-value	*F crit***
Between Groups	8897.143	6	148.285	148.285	1.07E-33	2.445259
Within Groups	28	28	1			
Total	8925.143	34				

§ Sum of squares

¥ Mean of squares

**Analysis showed that F> F crit (critical value). Therefore the null hypothesis was rejected and it was concluded that the variables (doubling time) are significantly different.

5.2.2. Morphological Characteristics of the *Bacillus* strains

5.2.2.1. *Bacillus* strains of *A. submarginata* Arc01, Arc02, Arc03, Arc04, Arc05, Arc06 and Arc07

All the isolates of *Bacillus* strains (Arc01-Arc07) from *A. submarginata* had rod shaped vegetative body, they were found to be gram positive, facultatively anaerobic, endospore forming, catalase positive and could produce acid from glucose and were highly motile. All these characteristics were similar to the characteristics of the members of genus *Bacillus* (Sneath, 1986). Further, all the morphological characteristics of the bacterial isolates (Arc01, Arc02, Arc03) such as colony morphology (Fig.5.7A, B, C & D), vegetative body structure, spore-shape (Fig. 5.8A, B, C & D), presence of parasporal crystals (Fig. 5.9A, B, C & D) were found to be similar to *Bacillus thuringiensis* (*Bt*) (Assaeedi et al., 2011). A comparison of morphological characteristics of the isolated strains with reference strain *Bacillus thuringiensis kurstaki* (*Btk*) is given in Table 5.7.

5.2.2.2. *Bacillus* strains of *A. bipunctata*: Ab01, Ab02, Ab03, Ab04, Ab05, Ab06, Ab07, Ab08, Ab09 and Ab10

All the morphological characteristics of *Bacillus* strains (Ab01, Ab02, Ab03 and Ab04) of *A. bipunctata* such as colony morphology (Fig. 5.10 A, B, C & D) vegetative body structure, spore-shape (Fig. 5.11 A, B, C & D), presence of parasporal crystals (Fig. 5.12 A, B, C & D) were found to be similar to *Bt* and the strains were comparable to the reference strain *Btk* (Table 5.10).

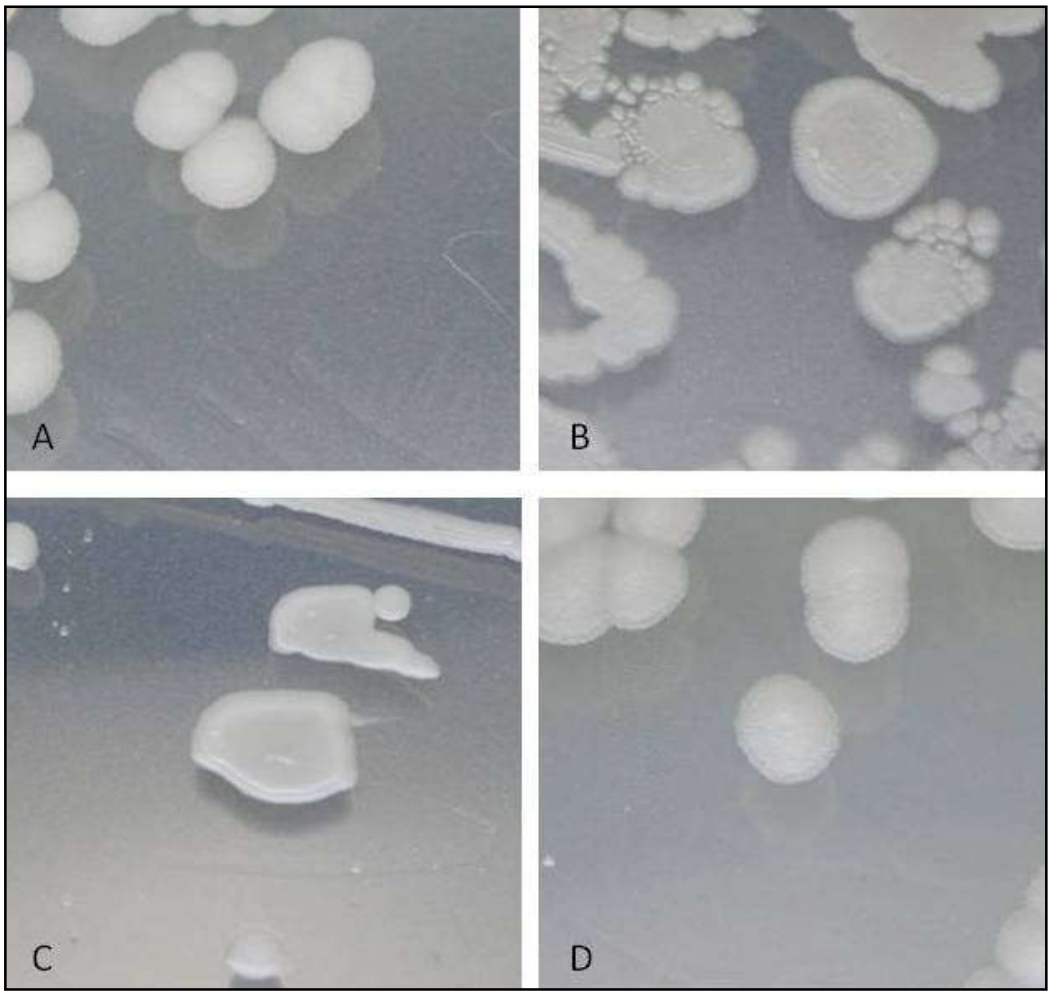


Fig. 5.7: Colony morphology of strains of *Bacillus*:- A) *Btk* (reference); B) Arc01; C) Arc02; D) Arc03.

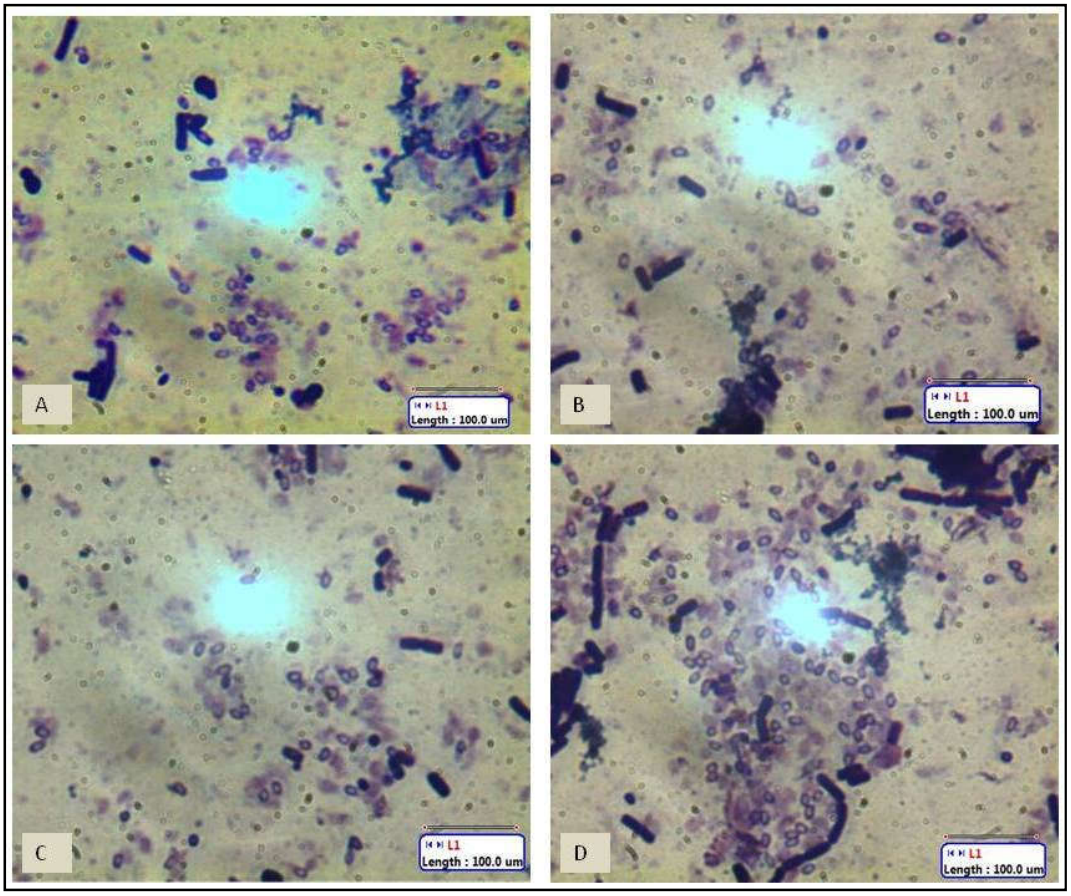


Fig. 5.8: Gram stained microphotographs (Vegetative cell, spore and crystal) of *Bacillus* strains:- A) *Btk* (reference); B) Arc01; C) Arc02; D) Arc03.

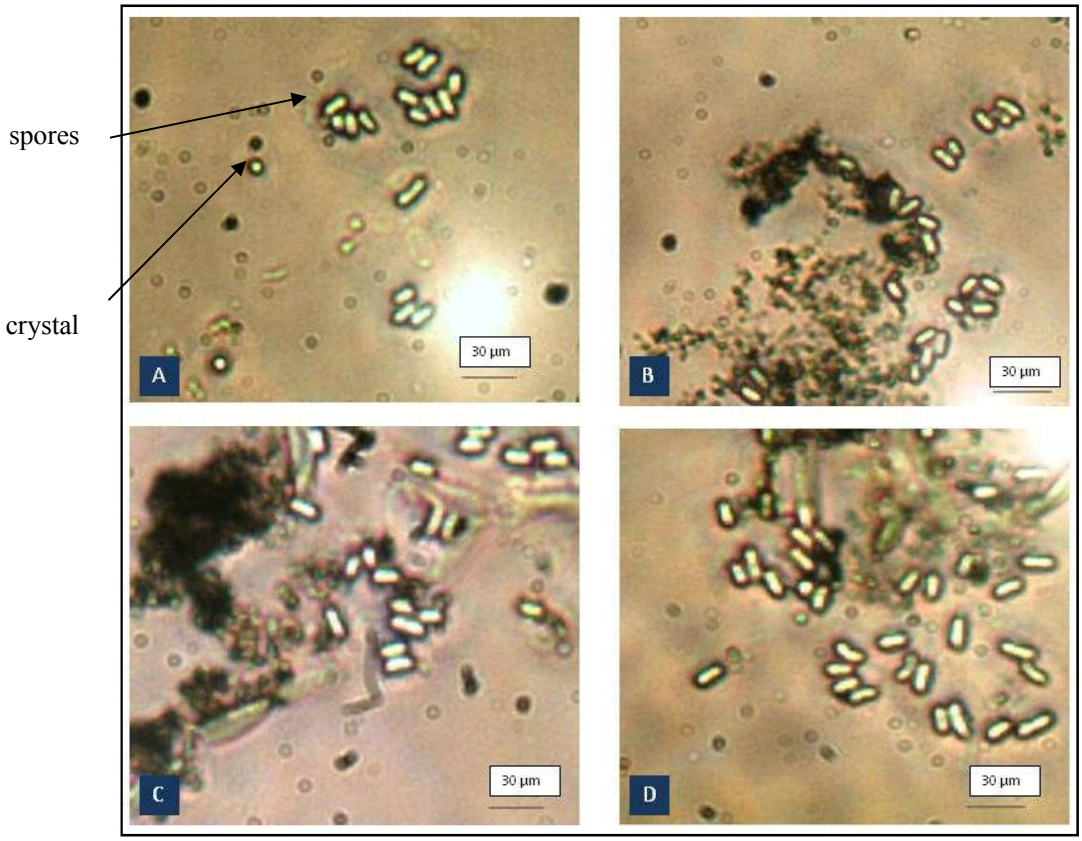


Fig. 5.9: Phase contrast microphotographs (Spore and crystal) of *Bacillus* Strains:- A) *Btk* (reference); B) Arc01; C) Arc02; D) Arc03.

Table 5.9: Morphological characteristics of the *Bacillus* strains of *A. submarginata* (Arc01-Arc07) and reference strain *Btk*.

Morphological Characteristics	*Bacillus thuringiensis kurstaki* (*Btk*)	Arc01	Arc02	Arc03	Arc 04	Arc 05	Arc 06	Arc 07
Shape of Vegetative cell	R	R	R	R	R	R	R	R
Chains of cells	+	+	+	+	+	+	+	+
Motility	HM	HM	HM	HM	HM	HM	HM	HM
Cell length > 3µm	+	+	+	+	+	+	+	+
Spore position and shape	VX	VX	VX	VX	VX	VX	VX	VX
Swelling of cell body by spore	-	-	-	-	-	-	-	-
Crystal protein structure	Bipyramidal	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical
Gram staining	+	+	+	+	+	+	+	+
Growth at 50°C	-	-	-	-	-	-	-	-
Growth at 10% NaCl	-	-	-	-	-	-	-	-
Anaerobic growth	+	+	+	+	+	+	+	+
Colony shape and configuration	Circular	Irregular	Circular	Circular	Rhizoid	Irregular	Circular	Fried egg
Colony texture	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Margin and elevation	Entire	Undulate	Entire	Entire	Entire	Undulate	Entire	Entire
Density/opacity	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
Pigmentation	White	White	White	White	White	White	White	White

R -Rod shaped; HM- Highly motile; V- spore central/subterminal; X- spore oval/ellipsoidal

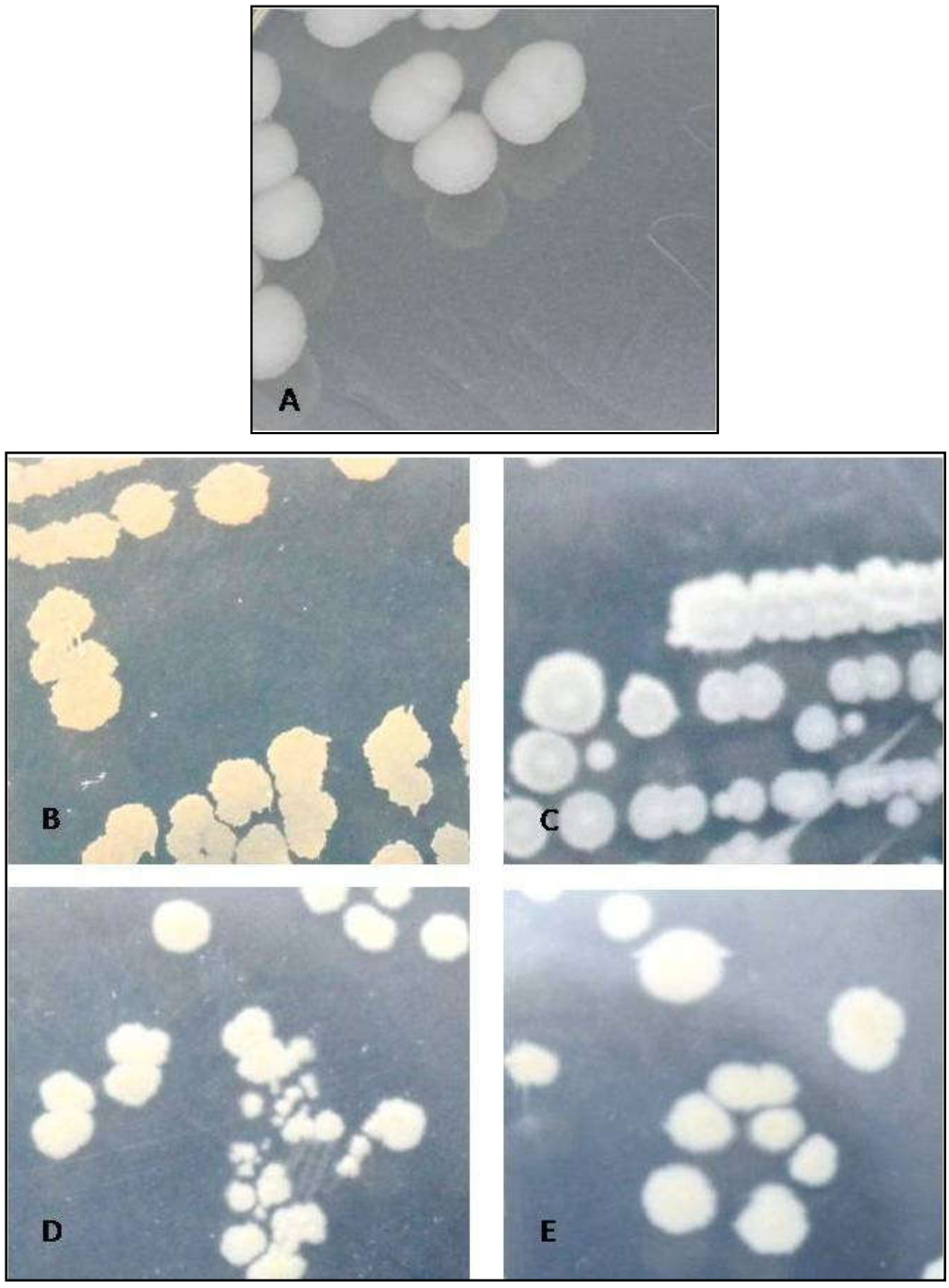


Fig. 5.10: Colony morphology of strains of *Bacillus* :- A) *Btk* (reference); B) Ab01; C) Ab02; D) Ab03; E) Ab04.

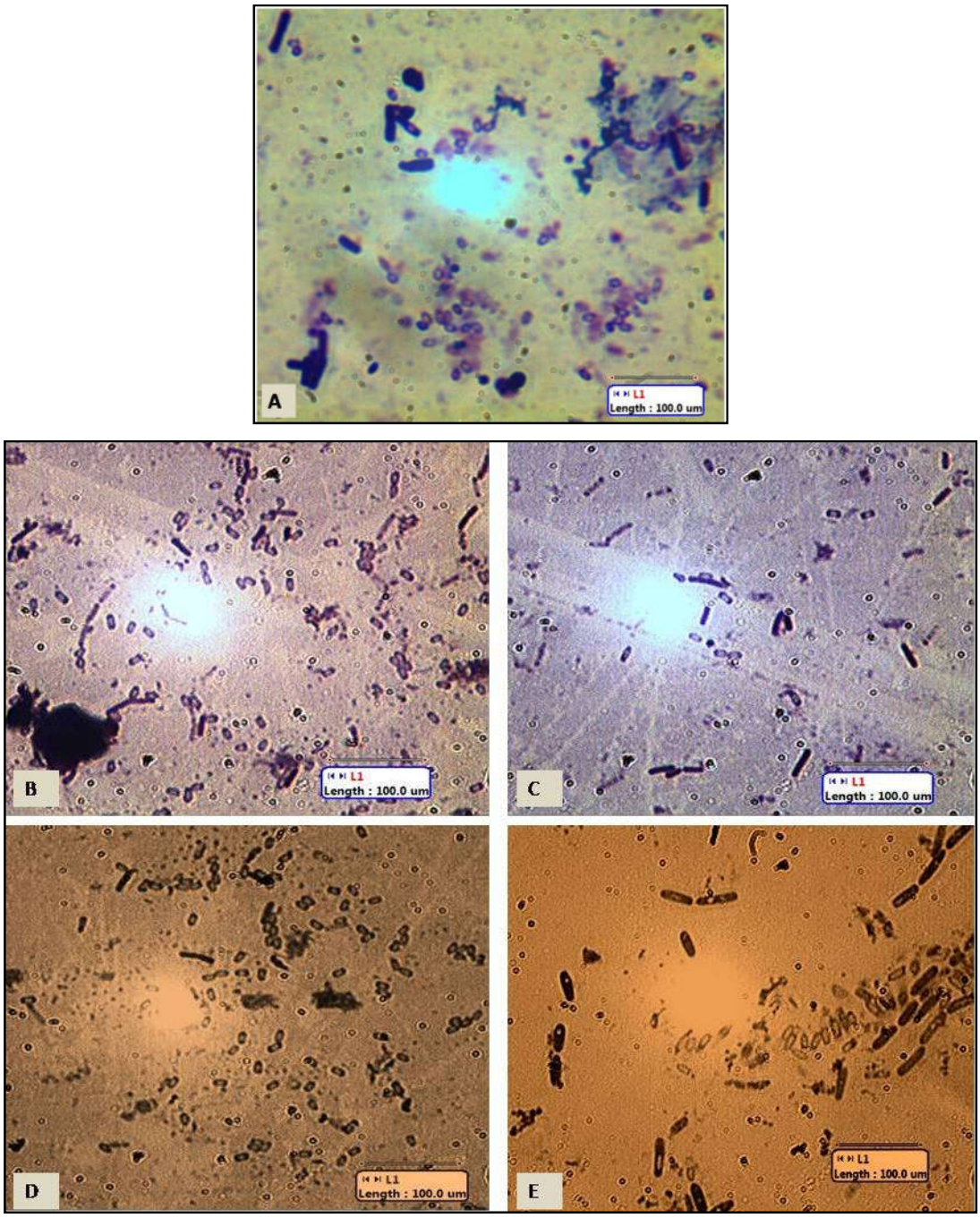


Fig. 5.11: Gram stained microphotographs (Vegetative cell, spore and crystal) of *Bacillus* strains:- A) of *Btk* (reference); B) Ab01; C) Ab02; D) Ab03 and D) Ab04.

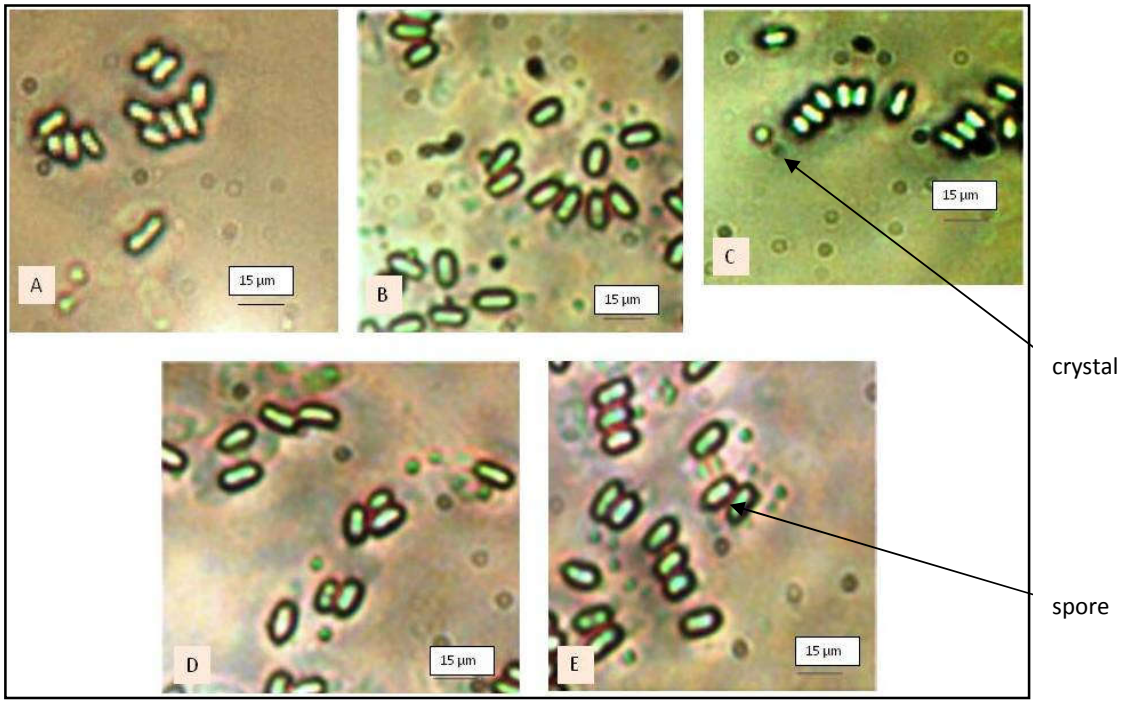


Fig. 5.12: Phase contrast microphotograph (Spore and crystal) of *Bacillus* strains:- A) *Btk* (reference); B) Ab01; C) Ab02; D) Ab03 and D) Ab04.

Table 5.10: Morphological characteristics of the bacterial isolates of *A. bipunctata* (Ab01-Ab10) and reference strain *Btk*.

Morphological Characteristics	*Bacillus thuringiensis kurstaki* (*Btk*)	Ab01	Ab02	Ab03	Ab04	Ab05	Ab06	Ab07	Ab08	Ab09	Ab10
Shape of Vegetative cell	R	R	R	R	R	R	R	R	R	R	R
Chains of cells	+	+	+	+	+	+	+	+	+	+	+
Motility	HM	HM	HM	HM	HM	HM	HM	HM	HM	HM	HM
Cell length > 3µm	+	+	+	+	+	+	+	+	+	+	+
Spore position and shape	VX	VX	VX	VX	VX	VX	VX	VX	VX	VX	VX
Swelling of cell body by spore	-	-	-	-	-	-	-	-	-	-	-
Crystal protein structure	Bipyramidal	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical	Spherical
Gram staining	+	+	+	+	+	+	+	+	+	+	+
Growth at 50°C	-	-	-	-	-	-	-	-	-	-	-
Growth at 10% NaCl	-	-	-	-	-	-	-	-	-	-	-
Anaerobic growth	+	+	+	+	+	+	+	+	+	+	+
Colony shape and configuration	Circular	Circular	Irregular	Circular	Circular	Circular	Irregular	Circular	Circular	Fried egg	Circular
Colony texture	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Margin and elevation	Entire	Entire	Undulate	Entire	Entire	Entire	Undulate	Entire	Entire	Entire	Entire
Density/opacity	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
Pigmentation	White	Cream	White	White	White	Cream	White	White	White	White	White

R -Rod shaped; HM- Highly motile; V- spore central/subterminal; X- spore oval/ellipsoid

5.2.2.3. *Bacillus* strains of *O. postica:* Org2A, Org6A, Org1, Org3, Org5 and Org5A

All the morphological characteristics of *Bacillus* strains (Org2A and Org6A) such as colony morphology (Fig. 5.13 A, B, C & D) vegetative body structure, spore-shape (Fig. 5.14 A, B, C & D), presence of parasporal crystals (Fig. 5.15 A, B, C & D) were found to be similar to *Bt* and were comparable to reference strain *Btk* (Table 5.11).

5.2.3. Biochemical characteristics of the *Bacillus* strains

5.2.3.1. *Bacillus* strains of *A. submarginata*: Arc01, Arc02, Arc03, Arc04, Arc05, Arc06 and Arc07

Biochemical characteristics of Arc01 showed positive reactions for lysine decarboxylase, ornithin decarboxylase, Voges-Proskaur, citrate utilization, nitrate reduction and utilization of trehalose and glucose. It showed differences with *Btk* in ONPG test, and in utilization of citrate, arabinose, xylose, cellobios, melibiose and saccharose. Arc02 strain showed positive reactions for lysine utilization, ornithin utilization, citrate utilization, malonate utilization, esculin hydrolysis, rhamnose, cellobiose, raffinose and glucose utilization, while differences with *Btk* were observed in ONPG test, V-P test, nitrate reduction, esculin hydrolysis and in utilization of citrate, malonate, arabinose, xylose, rhamnose, melibiose, saccharose, raffinose and trehalose. Likewise, Arc03 strain showed positive reaction in nitrate reduction, H_2S production, V-P test, esculin hydrolysis and utilization of citrate, saccharose, trehalose and glucose. It showed differences with *Btk* in ONPG test, H_2S production, esculin hydrolysis and utilization of lysine, ornithin, citrate, malonate, arabinose, xylose, rhamnose and melibios. When tested for startch hydrolysis, casein hydrolysis and catalase test all found to be positive except Arc02 which was negetive for casein

hydrolysis. The strain Arc04 showed positive reaction in lysine decarboxylase, ornithin decarboxylase, urease, Voges-Proskaur and in utilization of trehalose and glucose, but differed from *Btk* in ONPG, urease and nitrate test. Further, it showed differences in utilization of arabinose, xylose, cellobiose, melibiose, saccharose and lactose. Strain Arc05 showed positive reaction in lysine decarboxylase, ornithin decarboxylase, nitrate reduction,Voges-Proskaur, and urease tests, and in utilization of citrate, saccharose, trehalose and glucose, however, showed differences with *Btk* in ONPG, and urease tests, and in utilization of citrate, arabinose, xylose, cellobiose, melibiose and lactose. On the other hand, Arc06 strain was positive for ONPG, lysine decarboxylase, ornithin decarboxylase, urease, nitrate reduction, esculin hydrolysis and Voges-Proskaur tests, and in utilization of citrate, malonate, xylose, cellobiose, melibiose, saccharose, raffinose, trehalose and glucose. It showed differences with *Btk* in urease and esculin hydrolysis tests, and in utilization of citrate, malonate, arabinose, raffinose and lactose. The strain Arc07, was positive for ONPG, lysine decarboxylase, ornithin decarboxylase, urease, nitrate reduction, esculin hydrolysis and Voges-Proskaur tests and in utilization of citrate, malonate, xylose, arabinose, melibiose, saccharose, raffinose and trehalose. It showed differences with *Btk* in urease and esculin hydrolysis tests and in utilization of citrate, malonate, cellobiose, raffinose, glucose and lactose. When tested for starch hydrolysis, casein hydrolysis and catalase tests, all the strains were found to be positive except Arc02 which was negative to casein hydrolysis In comparision to *Btk*, *Bacillus* strains Arc01-07 of *A. submarginata* showed either positive (+) or negative (-) reactions for different tests used for Biochemical characterization of bacteria (Table 5.12).

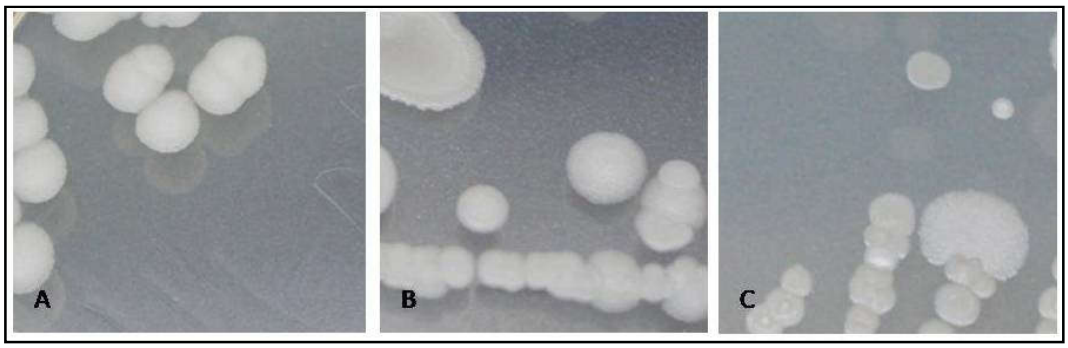


Fig. 5.13: Colony morphology of strains of *Bacillus*:- A) *Btk* (reference); B) Org 2A; C) Org 6A.

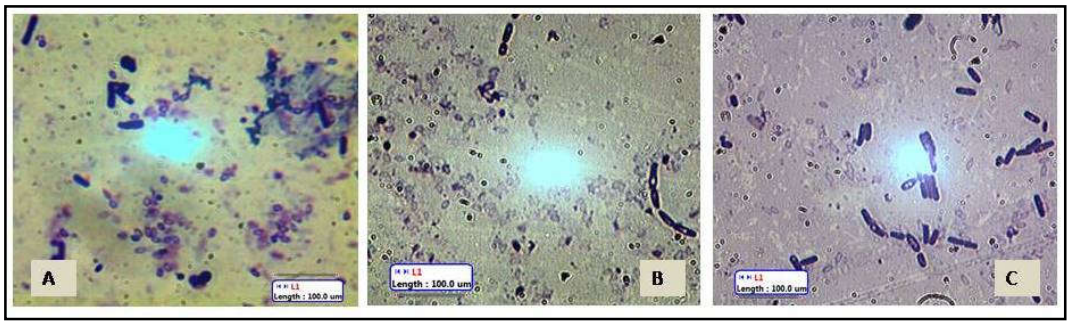


Fig. 5.14: Gram stained microphotographs (Vegetative cell, spore and crystal) :- A) *Btk* (reference); B) Org 2A; C) Org 6A.

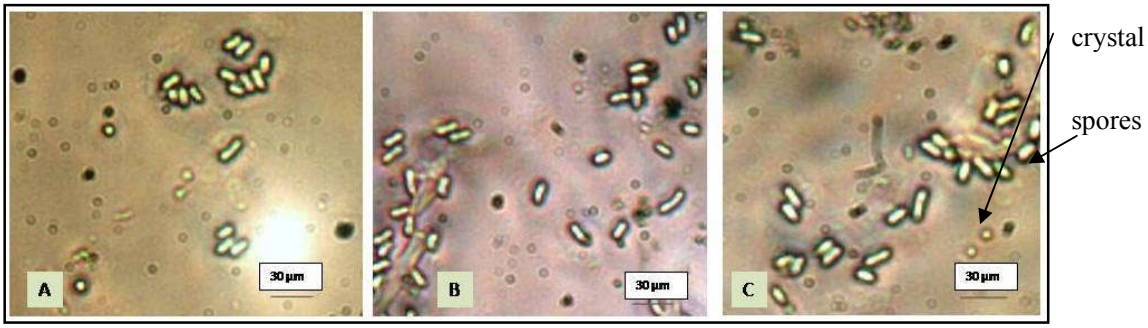


Fig. 5.15: Phase contrast microphotograph (Spore and crystal):- A) of *Btk* (reference); B) Org 2A; C) Org 6A.

Table 5.11: Morphological characteristics of the bacterial isolates of *O. postica* (Org 2A- Org5A) and reference strain *Btk*.

Morphological Characteristics	*Bacillus thuringiensis kurstaki* (*Btk*)	Org 2A	Org 6A	Org 1	Org 3	Org 5	Org 5A
Shape of Vegetative cell	R	R	R	R	R	R	R
Chains of cells	+	+	+	+	+	+	+
Motility	HM	HM	HM	HM	HM	HM	HM
Cell length > 3µm	+	+	+	+	+	+	+
Spore position and shape	VX	VX	VX	VX	VX	VX	VX
Swelling of cell body by spore	-	-	-	-	-	-	-
Crystal protein structure	Bipyramidal	Oval	Oval	Cubic	Spherical	Spherical	Spherical
Gram staining	+	+	+	+	+	+	+
Growth at 50°C	-	-	-	-	-	-	-
Growth at 10% NaCl	-	-	-	-	-	-	-
Anaerobic growth	+	+	+	+	+	+	+
Colony shape and configuration	Circular	Circular	Circular	Irregular	Circular	Rhizoid	Circular
Colony texture	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Margin and elevation	Entire	Entire	Entire	Undulate	Entire	Entire	Entire
Density/opacity	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
Pigmentation	White	White	White	Cream	White	Cream	White

R -Rod shaped; HM- Highly motile; V- spore central/subterminal; X- spore oval/ellipsoidal

lysine decarboxylase, ornithin decarboxylase, urease, Voges-Proskaur and in utilization of trehalose and glucose, but differed from *Btk* in ONPG, urease and nitrate test. Further, it showed differences in utilization of arabinose, xylose, cellobiose, melibiose, saccharose and lactose. Strain Arc05 showed positive reaction in lysine decarboxylase, ornithin decarboxylase, nitrate reduction,

Voges-Proskaur, and urease tests, and in utilization of citrate, saccharose, trehalose and glucose, however, showed differences with *Btk* in ONPG, and urease tests, and in utilization of citrate, arabinose, xylose, cellobiose, melibiose and lactose. On the other hand, Arc06 strain was positive for ONPG, lysine decarboxylase, ornithin decarboxylase, urease, nitrate reduction, esculin hydrolysis and Voges-Proskaur tests, and in utilization of citrate, malonate, xylose, cellobiose, melibiose, saccharose, raffinose, trehalose and glucose. It showed differences with *Btk* in urease and esculin hydrolysis tests, and in utilization of citrate, malonate, arabinose, raffinose and lactose. The strain Arc07, was positive for ONPG, lysine decarboxylase, ornithin decarboxylase, urease, nitrate reduction, esculin hydrolysis and Voges-Proskaur tests and in utilization of citrate, malonate, xylose, arabinose, melibiose, saccharose, raffinose and trehalose. It showed differences with *Btk* in urease and esculin hydrolysis tests and in utilization of citrate, malonate, cellobiose, raffinose, glucose and lactose. When tested for starch hydrolysis, casein hydrolysis and catalase tests, all the strains were found to be positive except Arc02 which was negative to casein hydrolysis In comparision to *Btk*, *Bacillus* strains Arc01-07 of *A. submarginata* showed either positive (+) or negative (-) reactions for different tests used for Biochemical characterization of bacteria (Table 5.12). The similarity coefficient among the bacterial isolates of *A. submarginata* (Arc01-Arc07) varied from 0.50 (50%) to 0.958 (95.8%). The lowest level of similarity 0.50 (50%) was recorded

between *Btk* and Arc02, whereas highest similarity of 0.958 (95.8%) was found between Arc01 and Arc05 (Table 5.13).

5.2.3.2. *Bacillus* strains of *A. bipunctata*: Ab01, Ab02, Ab03, Ab04, Ab05, Ab06, Ab07, Ab08, Ab09

Biochemical characteristics of Ab01 strain showed positive reactions for lysine decarboxylase, ornithin decarboxylase, oxidase tests, esculin hydorlysis and in utilization of citrate, arabinose, xylose, adonitol, cellobios, melibios, sacchrose, raffinose and lactose. It showed differences with *Btk* in ONPG, nitrate reduction and esculin hydrolysis tests and utilization of citrate, adonitol, raffinose, trehalose and glucose. Strain Ab02 showed positive reactions for nitrate reduction, esculin hydrolysis and in utilization of xylose, adonitol, rhamnose, cellobios, melibios, raffinose and lactose. It showed differences with *Btk* in ONPG, esculin and nitrate reduction tests and in utilization of adonitol, rhamnose, saccharose, raffinose, trehalose and glucose. The strain Ab03 was found to be positive for urease, nitrate reduction, esculin hydrolysis and in utilization of citrate, arabinose, xylose, adonitol, rhamnose, cellobios, melibios raffinose and lactose, while differences with *Btk* were observed in ONPG, lysine and ornithin decarboxylation, urease, esculin hydrolysis tests and in utilization of citrate, adonitol, rhamnose, saccharose, raffinose, trehalose and glucose. The strain Ab04 was positive for nitrate reduction, V-P test and in utilization of arabinose, xylose, adonitol, rhamnose, and lactose. It showed differences with *Btk* in ONPG, lysine and ornithin decarboxylation, urease, and in utilization of adonitol, rhamnose, cellobios, melibios, saccharose, trehalose and glucose.

Table 5.12: Biochemical characteristics of *Btk* (reference), Arc01, Arc02, Arc03, Arc04, Arc05, Arc06 and Arc07.

Biochemical tests	*Btk*	Arc 01	Arc 02	Arc 03	Arc04	Arc05	Arc06	Arc07
ONPG	+	-	-	-	-	-	+	+
Lysine decarboxylase	+	+	+	-	+	+	-	+
Ornithin decarboxylase	+	+	+	-	+	+	+	+
Urease	-	-	-	-	+	+	+	+
Phenylalanine deamination	-	-	-	-	-	-	-	-
Nitrate reduction	+	+	-	+	-	+	+	+
H_2S production	-	-	-	+	-	-	-	-
Citrate utilization	-	+	+	+	-	+	+	+
V-P Test	+	+	-	+	+	+	+	+
Methyl red	-	-	-	-	-	-	-	-
Indole	-	-	-	-	-	-	-	-
Malonate	-	-	+	+	-	-	+	+
Esculin hydrolysis	-	-	+	+	-	-	+	+
Arabinose	+	-	-	-	-	-	-	+
Xylose	+	-	-	-	-	-	+	+
Adonitol	-	-	-	-	-	-	-	-
Rhamnose	-	-	+	-	-	-	-	-
Cellobiose	+	-	+	-	-	-	+	-
Melibiose	+	-	-	-	-	-	+	+
Saccharose	+	-	-	+	-	+	+	+
Raffinose	-	-	+	-	-	-	+	+
Trehalose	+	+	-	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	-
Lactose	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-
Starch hydrolysis	+	+	+	+	+	-	+	+
Casein hydrolysis	+	+	-	+	+	+	+	+
Catalase test	+	+	+	+	+	+	+	+

+ denotes positive test result; - denotes negative test result.

Table 5.13: Similarity coefficient among the bacterial isolates of *A. submarginata* (Arc01-Arc07) and *Btk* (reference).

	Btk	Arc01	Arc02	Arc03	Arc04	Arc05	Arc06	Arc07
Btk	1.00							
Arc01	0.75	1.00						
Arc02	0.50▲	0.708	1.00					
Arc03	0.583	0.73	0.625	1.00				
Arc04	0.666	0.916	0.666	0.666	1.00			
Arc05	0.708	0.958▪	0.625	0.791	0.708	1.00		
Arc06	0.75	0.666	0.666	0.666	0.625	0.791	1.00	
Arc07	0.708	0.625	0.541	0.708	0.583	0.708	0.916	1.00

▲ indicates lowest similarity between two strains

▪ indicates highest similarity between two strains

Ab05 showed positive reaction for ornithin decarboxylase, urease, phenylalanine deamination, nitrate reduction, H_2S production and esculin hydrolysis tests, and in utilization of citrate, malonate, arabinose, xylose, cellobiose, melibiose, saccharose, raffinose, trehalose, glucose and lactose, but differed with *Btk* in ONPG, lysine decarboxylase, urease, phenylalanine deamination, H_2S, V-P and esculin hydrolysis tests, and in utilization of citrate, malonate and raffinose. Ab06 showed positive reactions for urease, nitrate, methyl red and esculin hydrolysis tests, and in utilization of malonate, arabinose, xylose, cellobiose, melibiose, trehalose, glucose, raffinose and saccharose. It showed difference with *Btk* in ONPG, lysine decarboxylase, ornitin decarboxylase, urease, V-P, methyl red and esculin hydrolysis tests and in utilization of malonate, raffinose and lactose. Ab07 was found to be positive for ONPG, lysine decarboxylase, ornithin decarboxylase, urease, nitrate reduction, H_2S production, methyl red and esculin hydrolysis tests and in utilization of malonate, arabinose, xylose, adonitol, rhamnose, trehalose, cellobiose, melibiose, glucose and lactose. It showed differences with *Btk* in urease, H_2S, V-P, methyl red and esculin hydrolysis tests and in utilization of malonate, adonitol, rhamnose, saccharose and raffinose. Ab08 showed positive reactions for ONPG, lysine decarboxylase, ornithin decarboxylase, urease, nitrate and esculin hydrolysis tests and in utilization of citrate, malonate, arabinose, xylose, adonitol, rhamnose, cellobiose, melibiose, saccharose, raffinose, trehalose, glucose and lactose. It showed differences with *Btk* in urease, V-P and esculin hydrolysis tests and in utilization of citrate, malonate, adonitol, rhamnose and raffinose. The strain Ab09 was positive for nitrate, methyl red and esculin hydrolysis tests and for utilization of citrate, malonate, arabinose, xylose, cellobiose, melibiose, saccharose, trehalose, raffinose and glucose. It showed differences with *Btk* in ONPG, lysine decarboxylase, ornithin decarboxylase, V-P,

methyl red and esculin hydrolysis tests and in utilization of citrate, malonate, raffinose and lactose. Ab10 showed positive reaction for ornithin decarboxylase, urease, nitrate, methyl red and esculin hydrolysis tests and in utilization of citrate, malonate, arabinose, xylose, cellobiose, melibiose, saccharose, raffinose and glucose. It showed differences with *Btk* in ONPG, lysine decarboxylase, urease, V-P, methyl red and esculin hydrolysis tests and in utilization of citrate, malonate, raffinose, trehalose and lactose.

When tested for starch hydrolysis, casein hydrolysis and catalase tes,t all the strains were found to be positive except Ab02 and Ab10, which were negative to casein hydrolysis (Table 5.14). The similarity coefficient among the bacterial isolates of *A. bipunctata* (Ab01-Arc10), ranged from 0.52 (52%) between *Btk* and Ab02 to 0.88 (88%) between the pairs Ab02 & Ab04, Ab06 & Ab09, Ab06 & Ab10 (Table 5.15).

5.2.3.3. *Bacillus* strains of *O. postica*: Org01, Org2A, Org03, Org 05, Org5A and Org6A

In biochemical tests, Org 2A showed positive reaction for lysine carboxylase, urease, H_2S productin, V-P test, esculin hydrolysis and utilization of citrate, malonate, rhamnose, sccharose, trehalose, glucose and lactose, while it differed from *Btk* in ONPG test, ornithin decarboxylation, urease, nitrate reduction, H_2S production, esculin hydrolysis and utilization of lysine, citrate, malonate, arabinose, xylose, rhamnose and melibios. The strain Org 6A was found to be positive for lysine carboxylase, ornithin decarboxylase, urease, H_2S production, V-P test and utilization of citrate, malonate, arabinose, cellobios, melibiose, raffinose, and glucose. Org 6A differed from *Btk* in ONPG test, nitrate reduction, H_2S production and in utilization of citrate, malonate, saccharose, raffinose and trehalose.

Table 5.14: Biochemical characteristics of *Btk* (reference), Ab01, Ab02, Ab03, Ab04, Ab05, Ab06, Ab07, Ab08, Ab09 and Ab10.

Biochemical tests	*Btk*	Ab01	Ab02	Ab03	Ab04	Ab05	Ab06	Ab07	Ab08	Ab09	Ab10
ONPG	+	-	-	-	-	-	-	+	+	-	-
Lysine decarboxylase	+	+	-	-	-	-	-	+	+	-	-
Ornithin decarboxylase	+	+	-	-	-	+	-	+	+	-	+
Urease	-	-	-	+	-	+	+	+	+	-	+
Phenylalanine deamination	-	-	-	-	-	+	-	-	-	-	-
Nitrate reduction	+	-	+	+	+	+	+	+	+	+	+
H_2S production	-	-	-	-	-	+	-	+	-	-	-
Citrate utilization	-	+	-	+	-	+	-	-	+	+	+
V-P Test	+	+	+	+	+	+	+	+	+	+	+
Methyl red	-	-	-	-	-	-	+	+	-	+	+
Indole	-	-	-	-	-	-	-	-	-	-	-
Malonate	-	-	-	-	-	+	+	+	+	-	+
Esculin hydrolysis	-	+	+	+	+	+	+	+	+	+	+
Arabinose	+	+	-	+	+	+	+	-	+	+	+
Xylose	+	+	+	+	+	+	+	+	+	+	+
Adonitol	-	+	+	+	+	-	-	+	+	-	-
Rhamnose	-	-	+	+	+	-	-	+	+	-	-
Cellobiose	+	+	+	+	-	+	+	+	+	+	+
Melibiose	+	+	+	+	-	+	+	+	+	+	+
Saccharose	+	+	-	+	-	+	+	-	+	+	+
Raffinose	-	+	+	+	-	+	+	+	+	+	+
Trehalose	+	-	-	-	-	+	+	+	+	+	-
Glucose	+	-	-	-	-	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	-	+	+	-	-
Oxidase	-	-	-	-	-	-	-	-	-	-	-
Starch hydrolysis	+	+	+	+	+	+	-	+	+	+	+
Casein hydrolysis	+	+	-	+	+	+	+	+	+	+	-
Catalase test	+	+	+	+	+	+	+	-	-	+	+

+ denotes positive test result; - denotes negative test result.

Table 5.15: Similarity coefficient among the bacterial isolates of *A. bipunctata* (Ab01-Ab10) and *Btk* (reference).

	Btk	Ab01	Ab02	Ab03	Ab04	Ab05	Ab06	Ab07	Ab08	Ab09	Ab10
Btk	1.00										
Ab01	0.68	1.00									
Ab02	0.52▲	0.72	1.00								
Ab03	0.56	0.80	0.84	1.00							
Ab04	0.56	0.64	0.88▪	0.76	1.00						
Ab05	0.64	0.666	0.541	0.708	0.458	1.00					
Ab06	0.6	0.583	0.625	0.708	0.541	0.76	1.00				
Ab07	0.6	0.541	0.625	0.583	0.50	0.64	0.64	1.00			
Ab08	0.72	0.75	0.625	0.791	0.541	0.76	0.68	0.80	1.00		
Ab09	0.68	0.708	0.666	0.75	0.541	0.72	0.88▪	0.52	0.72	1.00	
Ab10	0.6	0.708	0.583	0.75	0.50	0.72	0.88▪	0.60	0.72	0.84	1.00

▲ indicates lowest similarity between two strains

▪ indicates highest similarity between two strains

Similarly, Org01 showed positive reaction for ONPG, lysine decarboxylase, ornithin decarboxylase, nitrate reduction, H_2S production, V-P test, esculine hydrolysis and utilization of arabinoe, cellobios, melibios, sccharose, trehalose, glucose and lactose and showed differences with *Btk* in H_2S production, esculine hydrolysis and utilization of xylose and lactose. Org03 showed positive reaction for nitrate reduction, V-P test, esculine hydrolysis and utilization of citrate, arabinose, sccharose, trehalose, glucose and lactose. It showed diffrences with *Btk* in ONPG, lysine decarboxylase, esculine hydrolysis and utilization of citrate, xylose, cellobiose, mellibiose and lactose. The strain Org05 showed positive reaction for ONPG, phenylalanine deamination, V-P test, esculine hydrolysis and utilization of citrate, malonate, arabinose, xylose, cellobios, sccharose, raffinose, trehalose, glucose and lactose differing from *Btk* in lysine decarboxylase, ornithin decaboxylase, phenylalanine deamination, nitrate reduction, esculine hydrolysis and utilization of citrate, malonate, melibiose, raffinose and lactose tests. Org5A showed positive reactions for ONPG, lysine decarboxylase, ornithin decarboxylase, urease, nitrate reduction, H_2S production, V-P test, esculine hydrolysis and utilization of citrate, arabinose, xylose, adonitol, rhamnose, cellobiose, melibiose, trehalose, glucose and lactose. It showed difference with *Btk* for urease, H_2S production, esculine hydrolysis and utilization of citrate, adonitol, rhamnose, sccharose and lactose. When tested for startch hydrolysis, casein hydrolysis and catalase test all the strains were positive to the above tests. (Table 5.16). The similarity coefficient among the bacterial isolates of *O. postica* (Org01-Org6A), varied from a lowest 0.44 (44%) between *Btk* and Org2A to a highest value of 0.84 (84%) between *Btk* and Org01 (Table 5.17).

Table 5.16: Biochemical characteristics of *Btk* (reference), Org 2A, Org 6A, Org04, Org03, Org05 and Org5A.

Biochemical tests	*Btk*	Org2A	Org6A	Org1	Org3	Org5	Org5A
ONPG	+	-	-	+	-	+	+
Lysine decarboxylase	+	+	+	+	-	-	+
Ornithin decarboxylase	+	-	+	+	-	-	+
Urease	-	+	+	-	-	-	+
Phenylalanine deamination	-	-	-	-	-	+	-
Nitrate reduction	+	-	-	+	+	-	+
H_2S production	-	+	+	+	-	-	+
Citrate utilization	-	+	+	-	+	+	+
V-P Test	+	+	+	+	+	+	+
Methyl red	-	-	-	-	-	-	-
Indole	-	-	-	-	-	-	-
Malonate	-	+	+	-	-	+	-
Esculin hydrolysis	-	+	-	+	+	+	+
Arabinose	+	-	+	+	+	+	+
Xylose	+	-	+	-	-	+	+
Adonitol	-	-	-	-	-	-	+
Rhamnose	-	+	-	-	-	-	+
Cellobiose	+	-	+	+	-	+	+
Melibiose	+	-	+	+	-	-	+
Saccharose	+	+	-	+	+	+	-
Raffinose	-	-	+	-	-	+	-
Trehalose	+	+	-	+	+	+	+
Glucose	+	+	+	+	+	+	+
Lactose	-	+	-	+	+	+	+
Oxidase	-	-	-	-	-	-	-
Starch hydrolysis	+	+	+	+	+	+	+
Casein hydrolysis	+	+	+	+	-	+	+
Catalase test	+	+	+	+	+	+	-

+ denotes positive test result; - denotes negative test result.

Table 5.17: Similarity coefficient among the bacterial isolates of *O. postica* (Org01-Org6A) and *Btk* (reference).

	Btk	Org2A	Org6A	Org01	Org03	Org05	Org5A
Btk	1.00						
Org2A	0.44▲	1.00					
Org6A	0.64	0.56	1.00				
Org01	0.84▪	0.58	0.708	1.00			
Org03	0.64	0.83	0.541	0.60	1.00		
Org05	0.60	0.58	0.708	0.60	0.72	1.00	
Org5A	0.68	0.58	0.708	0.76	0.56	0.52	1.00

▲ indicates lowest similarity between two strains

▪ indicates highest similarity between two strains

5.3 Sodium-dodecyl sulphate (SDS) polyacralamide gel electrophoresis (PAGE) of bacterial proteins

5.3.1 SDS-PAGE of Crystal protein

- ***Bacillus* strains: Arc01 and *Btk* (reference).**

When crystal protein (cry) composition was analysed by SDS-PAGE, difference in molecular weight was found between *Bacillus* strain Arc 01 and the *Btk* (Fig. 5.16). The strain Arc01 and *Btk* both showed four bands of cry proteins of 128, 81, 64 and 55.6 kDa were present in Arc01 and 76, 67, 56.6, 44.4 and 29.5 kDa were observed in *Btk*, which suggests variation in crystal protein profile of *Bacillus* strain Arc01 and the reference strain *Btk*.

- ***Bacillus* strains: Arc02, Arc03 and *Btk* (reference).**

When composition of crystal protein (cry) was analyzed by SDS-PAGE, Arc 02 showed five protein bands having the molecular weight 117.3, 75.1, 55.9, 39.1 and 29 kDa, which in case of *Btk* were 115.8, 75.1, 56.6, 44.4 and 29.5 kDa. Arc 03 also revealed five protein bands of 122.7, 85.8, 56.3, 46.8 and 29 kDa but they were different from Arc 02 and *Btk*. So, a sharp difference in banding pattern was found between Arc 02, Arc03 and *Btk*. Again variability in the crystal protein profile was seen in the *Bacillus* strains Arc02, Arc03 and reference strain *Btk*. Presence of more than one crystal protein band also suggests that these isolates may have newer crystal toxins different from one another and also with reference *Btk* (Fig. 5.17).

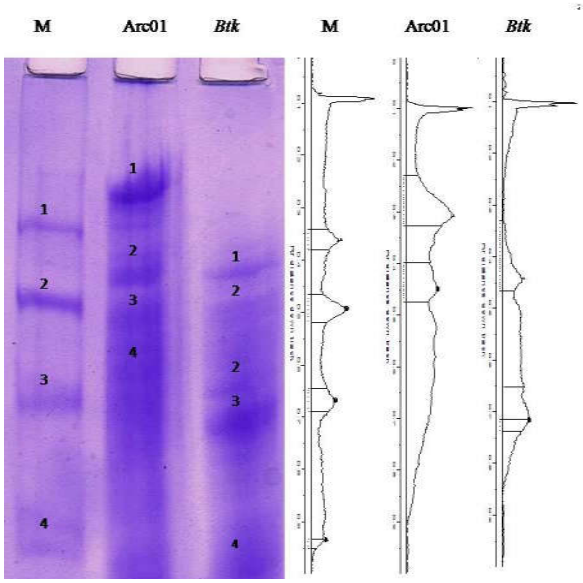


Fig. 5.16: SDS PAGE analysis of Whole body protein of Arc 01 and *Btk* (reference) [M: 97.4 kDa, 66 kDa, 43 kDa, 29 kDa; *Btk*: 76 kDa, 67 kDa, 56.6 kDa, 44.4 kDa, 29.5 kDa; Arc01: 128 kDa, 81 kDa, 64 kDa, 55.6 kDa] with corresponding pixel graph.

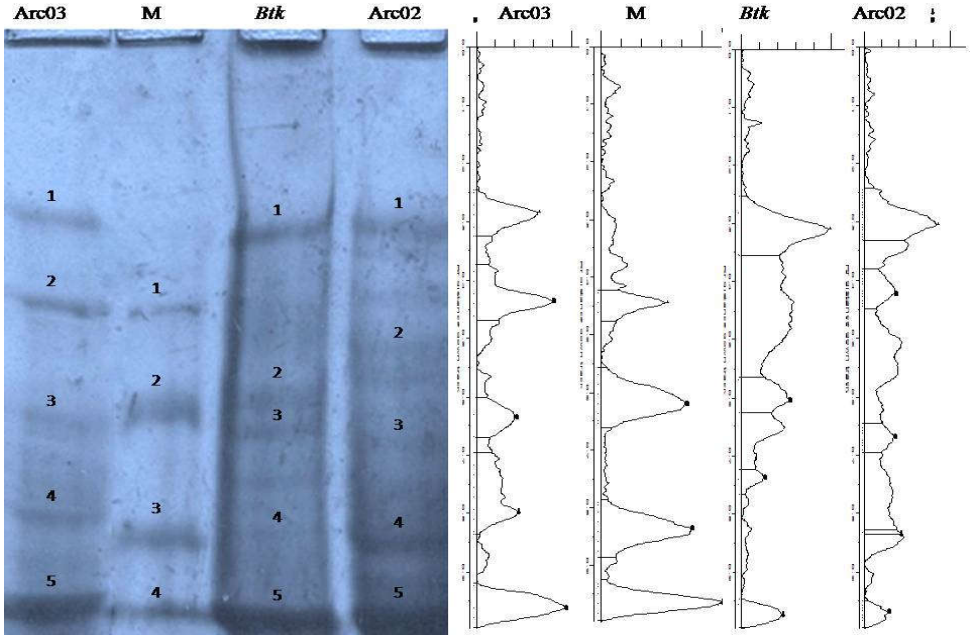


Fig. 5.17: SDS-PAGE of crystal protein of Arc02, Arc03 and *Btk* (reference) [Arc02: 117.3 kDa, 75.1 kDa, 55.9 kDa, 39.1 kDa, 29 kDa ; Arc03: 122.7 kDa, 85.8 kDa, 56.3 kDa, 46.8 kDa, 29 kDa; *Btk*: 115.8 kDa, 75.1 kDa, 56.6 kDa, 44.4 kDa, 29.5 kDa and Marker: 97.4 kDa, 66 kDa, 43 kDa, 29 kDa] with corresponding pixel graph.

- ***Bacillus* strains: Ab01, Ab02, Ab03, Ab04 and *Btk* (reference).**

When composition of cry protein was analyzed by SDS-PAGE for the *Bacillus* strain isolated from *A. bipunctata*, Ab01and Ab02 revealed four bands each of 88, 56.7 and 43.3, 28 kDa and 88.9, 54.5, 43.8 and 27.5 kDa, respectively. Therefore, these two strains had crystal protein bands of comparable molecular weight. Each of the Ab03, Ab04 and reference stain *Btk* strains showed five distinct bands, Ab03 had bands of 56.2, 43, 38.2, 31 and 28 kDa, whereas 129.8, 97, 56.5, 44 and 29.4 kDa bands were present in Ab04 and *Btk* revealed 128.4, 97, 56.6, 44.4 and 29.5kDa bands. All the *Bacillus* strains Ab01-04 differed in crystal protein composition from the reference strain *Btk,* suggesting variability in crystal protein composition present among them and with reference strain *Btk* (Fig. 5.18).

- ***Bacillus* strains: Org2A, Org6A and *Btk* (reference).**

Similarly, SDS-PAGE analysis of cry protein Org2A and Org 6A was also carried out. Both the strains, Org2A and Org6A showed one major protein band having the molecular weight 36.5 kDa and 57.7 kDa, respectively. In contrast, *Btk* had five bands of 110.7, 97, 56.6, 44.4 and 29.5 kDa (Fig. 5.19). Unlike other strains mentioned above these two strains possessed only one bands suggesting they have only specific crystal toxin. All the band profiles showed difference within and between the strains.

5.3.2 Qualitative and Quantitative assay of whole cellular proteins of the isolates

Comparison of the whole cell proteins of different *Bacillus* strains of *Bt* isolated from *A. submarginata*, *A. bipunctata* and *O. postica* showed that the protein amount varied from 1.094-2.356 mg/ml in overnight grown bacterial culture.

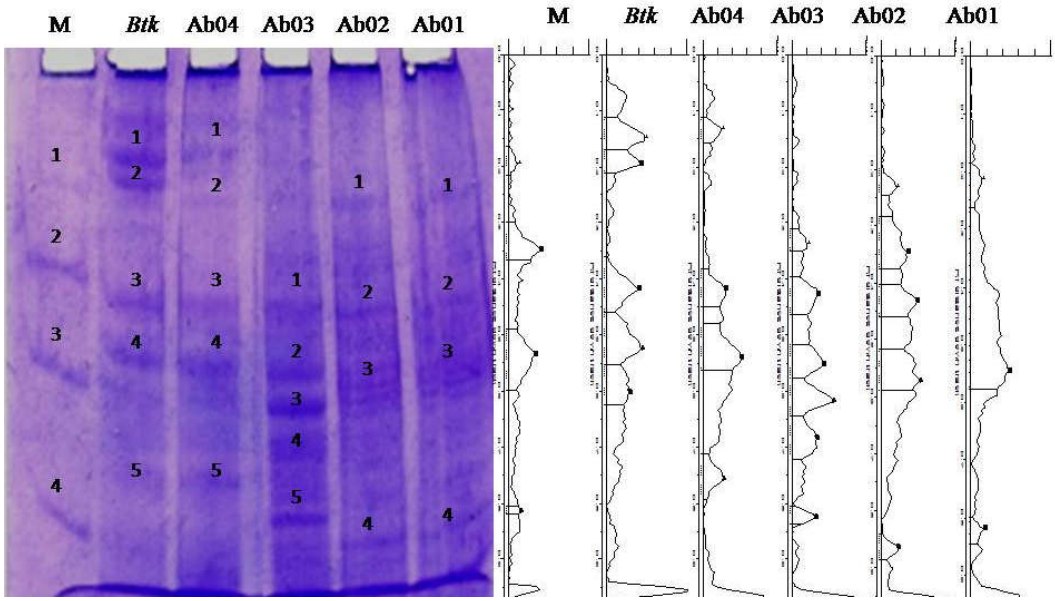


Fig. 5.18: SDS-PAGE of crystal proteins of *Bacillus* Ab01, Ab02, Ab03, Ab04 and *Btk* (reference) [Ab01: 88 kDa, 56.7 kDa,43.3kDa, 28 kDa; Ab02: 88.9 kDa, 54.5 kDa, 43.8 kDa, 27.5 kDa; Ab03: 56.2 kDa, 43 kDa, 38.2 kDa, 31 kDa, 28 kDa, Ab04: 129.8 kDa, 97 kDa, 56.5 kDa, 44 kDa, 29.5 kDa; *Btk*: 128.4 kDa, 97 kDa, 56.6 kDa, 44.4 kDa, 29.5 kDa; Marker: 97.4 kDa, 66 kDa, 43 kDa, 29 kDa] with corresponding pixel graph.

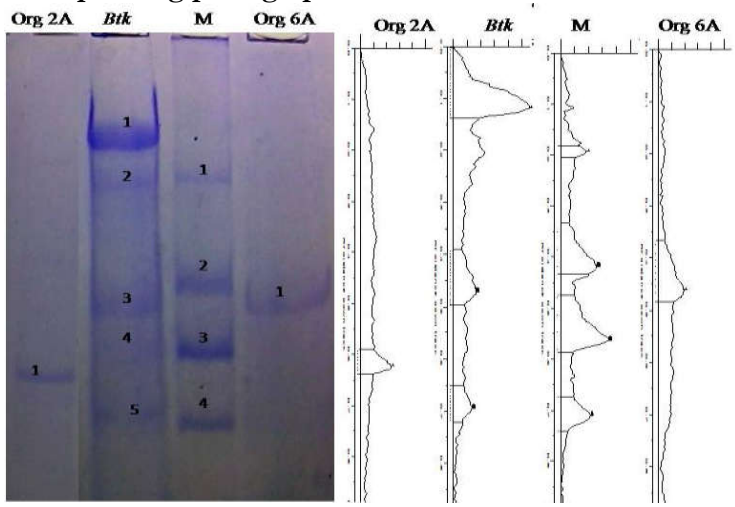


Fig. 5.19: SDS-PAGE of crystal protein of *Bacillus* sp Org 2A, Org 6A and *Btk* (reference) [Org2A: 36.5 kDa; Org6A: 57.7 kDa; *Btk*: 110.7 kDa, 97kDa, 56.6 kDa, 29.5 kDa; Marker: 97.4 kDa, 66 kDa, 43 kDa, 29 kDa] with corresponding pixel graph.

The amount of proteins were 1.346, 1.420, 2.356, 1.094, 1.175, 1.244, 2.103, 1.994, 1.920 mg/ml for Arc01, Arc02, Arc03, Ab01, Ab02, Ab03, Ab04, Org2A, Org6A, respectively, compared to 2.128 mg/ml in *Btk* (Table 5.18). It indicated that different strains varied in whole cellular protein concentration.

In SDS PAGE analysis, the total cellular proteins of ten *Bacillus* strains as well as reference strain (Fig. 5.20, 5.21, 5.22, 5.23) were separated into several protein bands ranging from 10 to 126 KDa (Tables 5.19). The electrophoregram (Scans of protein profiles) revealed that the protein profiles can be distinguished into different groups on the basis of the molecular mass, which were in the range of 31 to 97 kDa (Arc01), 27 to 126 kDa (Arc02), 10 to 124 kDa (Arc03), 41 to 54 kDa (Ab01), 40 to 85 kDa (Ab02), 40 to 98 kDa (Ab03), 29 to 110 kDa (Ab04), 31 to 78 kDa (Org2A) and 31 to 77 kDa (Org6A). In *Btk* (reference strain) molecular masses were in the range of 25- 110 kDa combining four gels (Table 5.18). The protein profiles of isolated *Bacillus* strains could be distinguished into three protein groups, i.e. i) the proteins of low molecular masses of 25 to 59 kDa ii) the proteins of moderate molecular masses of 60 to 85 kDa and iii) the proteins of high molecular masses of 110 to 128 kDa (Table 5.19).

Table 5.18: Quantification of whole cellular proteins (Arc01, Arc02, Arc03, Ab01, Ab02, Ab03, Ab04, Org2A, Org6A) and *Btk* (reference).

Quantitative (Spectrophotometer)				
Bacillus strains	Optical density (OD)(at 545 nm)	Protein amount(mg/ml)	No. of bands	Molecular Weight Range (kDa)
Arc01	0.267	1.346	4	31-97
Arc02	0.280	1.420	3	25.8-126.6
Arc03	0.442	2.356	8	10-124
Ab01	0.224	1.094	2	40.8-54.1
Ab02	0.238	1.175	3	40.5-85
Ab03	0.250	1.244	4	40.5-98
Ab04	0.398	2.103	5	29-110
Org 2A	0.379	1.994	4	31.2- 78.5
Org 6A	0.367	1.920	3	30.8- 76.8
Btk	0.403	2.128	8	25-110

Table 5.19: Protein groups of nine *Bacillus* strains (Arc01, Arc02, Arc03, Ab01, Ab02, Ab03, Ab04, Org2A, Org6A) and *Btk* (reference).

Molecular masses of protein groups			
Bt strains	Group I (25 -59 kDa)	Group II (60 -80 kDa)	Group III (81 -135 kDa)
Arc01	31, 42.7	65.5	97
Arc02	27.7, 43	-	126.6
Arc03	10, 18, 26.8, 42.9, 50.3,	60.4	85.5, 124
Ab01	40.8, 54.1	-	-
Ab02	40.5, 58.1	-	85
Ab03	40.4, 55.8	-	84.8, 98
Ab04	29, 40.8	60.4	81.4, 110
Org2A	31.2, 42.6, 51	78.5	-
Org6A	30.8, 50	76.8	-
Btk	25, 31, 41, 55, 58	67, 76	110

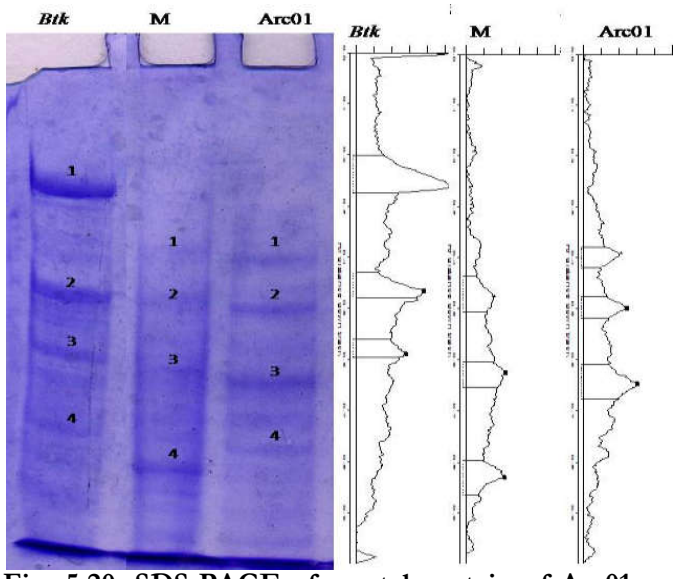


Fig. 5.20: SDS-PAGE of crystal protein of Arc01 and *Btk* (reference) [*Btk*: 115.8 kDa, 67 kDa, 45 kDa, 32 kDa; Arc01: 97 kDa, 65.5 kDa, 42.7 kDa, 31.2 kDa; M: 97.4 kDa, 66 kDa, 43 kDa, 29 kDa;] with corresponding pixel graph.

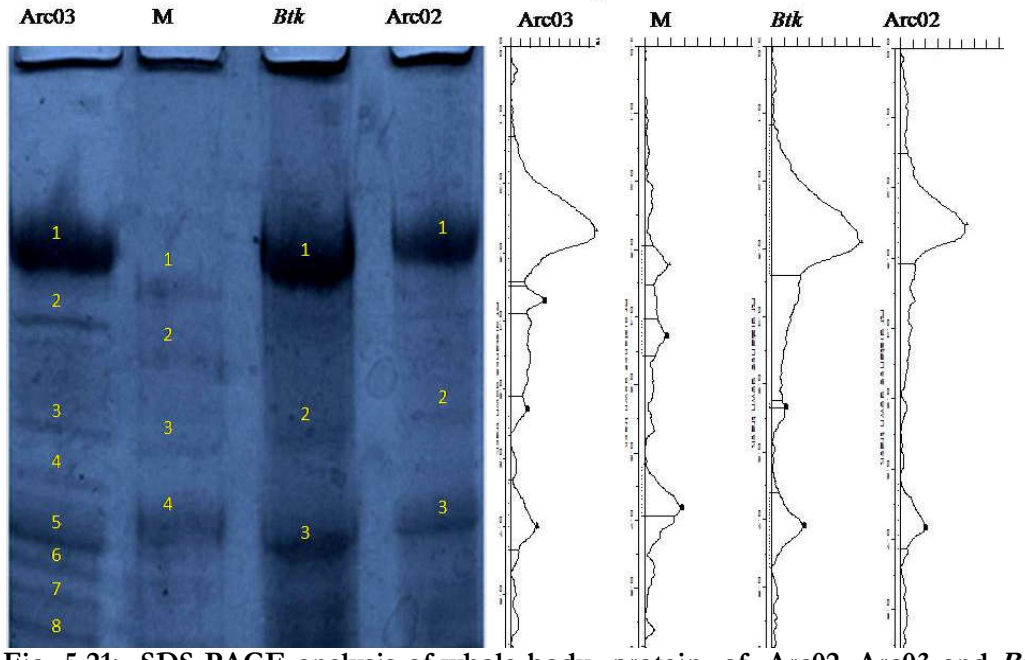


Fig. 5.21: SDS-PAGE analysis of whole body protein of Arc02, Arc03 and *Btk* (reference) [Arc02: 126.6 kDa, 43 kDa, 27.7 kDa; Arc03: 124 kDa, 85.8 kDa, 60.4 kDa, 50.3 kDa, 42.9 kDa, 26.8 kDa, 18 kDa, 10 kDa; *Btk*: 110.5 kDa, 55.3 kDa, 25.8 kDa and Marker: 97.4 kDa, 66 kDa, 43 kDa, 29 kDa.] with corresponding pixel graph.

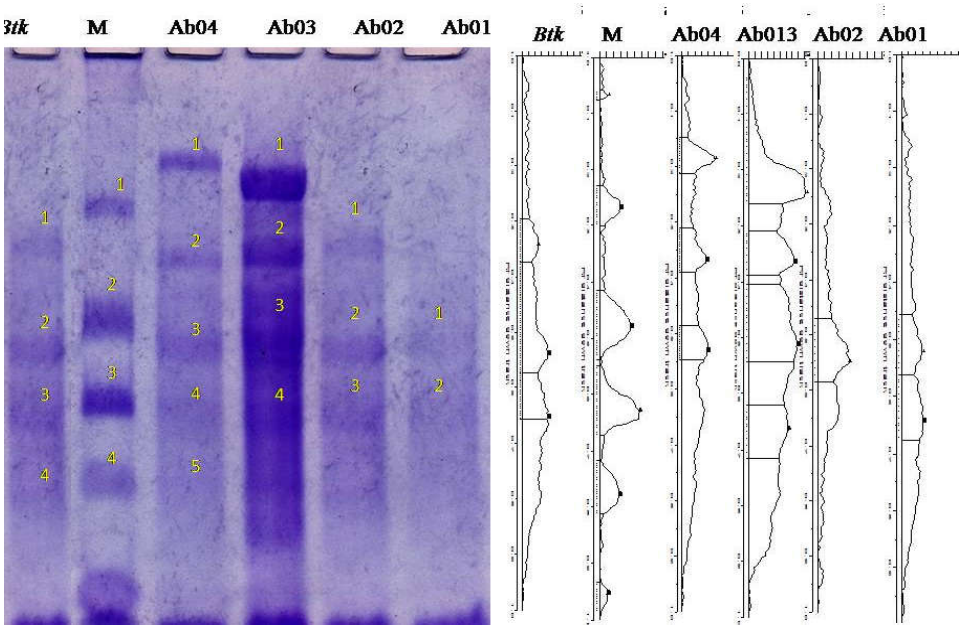


Fig. 5.22: SDS-PAGE of whole body protein of Ab01, Ab02, Ab03, Ab04 and *Btk* (reference) [Ab04: 110 kDa, 81.4 kDa, 60.4 kDa, 40.8kDa, 29 kDa; Ab03: 98 kDa, 84.8 kDa, 55.8 kDa, 40.5 kDa; Ab02: 85 kDa, 58.1 kDa, 40.5 kDa; Ab01: 54.1kDa, 40.8 kDa; *Btk*: 76.3 kDa, 58 kDa, 40.5 kDa, 25.8 kDa and Marker: 97.4 kDa, 66 kDa, 43 kDa, 29 kDa] with corresponding pixel graph.

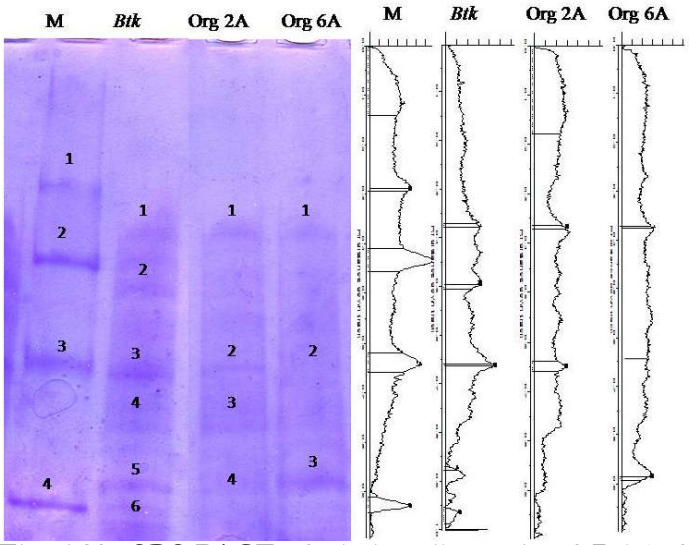


Fig. 5.23: SDS-PAGE of whole-cell protein of *Btk* (reference), Org 2A and Org 6A [Marker: 97.4 kDa, 66 kDa, 43 kDa, 29 kDa; *Btk*: 76.3 kDa, 58 kDa, 51 kDa, 41.9 kDa, 25.5 kDa; Org 2A: 78.5 kDa, 51 kDa, 42.6 kDa, 31.2 kDa; Org 6A: 76.8 kDa, 50 kDa, 30.8 kDa] with corresponding pixel graph.

5.4 Plasmid profiling of the bacterial strains:

- ***Bacillus* strains: Arc01, Arc02, Arc03 and *Btk* (reference).**

Plasmid profiling of *Bacillus* strains of *A. submarginata* (Arc01, Arc02 and Arc03) showed one major plasmid band with approximate molecular weight 20.7 kbp, 18.5 kbp and 18.1 kbp respectively, which were comparable to the reference strain *Btk* which had 19 kbp band but slightly differed in plasmid size among themselves and also with the *Btk* (Fig. 5.24).

- ***Bacillus* strains: Ab01, Ab02, Ab03, Ab04 and *Btk* (reference).**

Plasmid profiling of *Bacillus* of *A. bipunctata* (Ab01, Ab02, Ab03 and Ab04) and reference strain *Btk* showed one major plasmid band ranging from 16 to 19 kbp. Ab01 had a band of 16 kbp followed by 17 kbp band of Ab02, while Ab03 and Ab04 had 18.2 kbp and 19.4 kbp bands, respectively. *Btk* yielded a major band of 19 kbp. All the isolates differed among themselves and also with the *Btk* in their plasmid profile (Fig. 5.25).

- ***Bacillus* strains: Org 2A, org 6A and *Btk* (reference).**

Plasmid profiling of *Bacillus* of *O. postica* (Org2A and Org6A) and *Btk* showed one major plasmid band for each with molecular weight 20.5 kbp, 19.8 kbp and 19 kbp, respectively which differed among themselves and with the *Btk* as well (Fig 5.26).

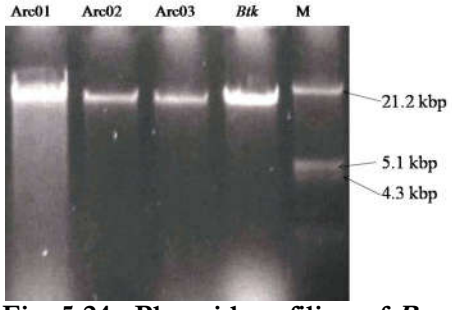


Fig. 5.24: Plasmid profiling of *Bacillus* sp. Arc01 [20.7 kbp], Arc02[18.5 kbp], Arc03[18.1 kbp] and *Btk* (reference)[19 kbp]with *Hind* III and *Eco* RI double digested λ DNA as Marker.

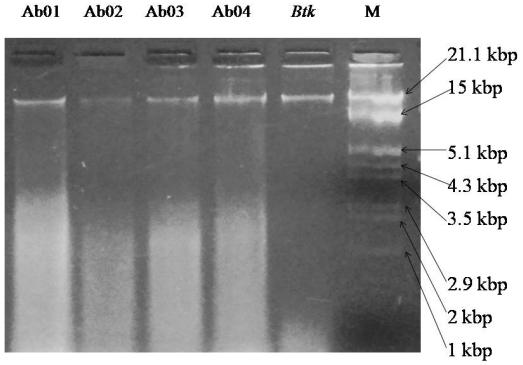


Fig. 5.25: Plasmid profiling of *Bacillus* sp. Ab01 [16 kbp], Ab02 [17 kbp], Ab03 [18.2 kbp], Ab04 [19.4 kbp] and *Btk* (reference)[19 kbp] with *Hind* III and *Eco* RI double digested λ DNA as Marker.

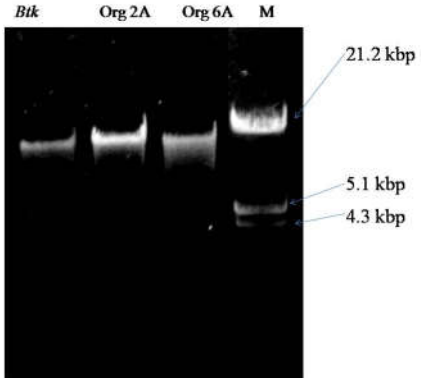


Fig. 5.26: Plasmid profiling of *Bacillus* sp. Org2A [20.5 kbp], Org6A [19.8 kbp] and *Btk* (reference)[19 kbp] with *Hind* III and *Eco* RI double digested λ DNA as Marker.

5.5 Bioassay

5.5.1 Rearing of the test insects in laboratory

5.5.1.1 Rearing of sporadic pests in laboratory

(i) *Arctornis submarginata* (Walker)

Systematic position

Phylum: Arthropoda

Class: Insecta

Order: Lepidoptera

Family: Lymantriidae

Genus: *Arctornis*

Species: *A. submarginata*

Moths of *A. submarginata* were collected from the tea plantations of Darjeeling Terai and the Dooars. They were then allowed to mate and lay eggs in sterilized plastic container, towelled with tissue paper. Fertilized eggs were then transferred in fresh containers for hatching. After hatching, the 1st instar caterpillars were transferred to small transparent plastic buckets and thoroughly washed fresh tea leaves (with petioles of young leaves submerged in water filled 2ml micro centrifuge tube) were provided as food to the neonates. The bucket was sanitized and food was changed regularly. At 3rd instar stage the caterpillars were transferred to bigger transparent buckets provided with mature tea leaves as food. Leaves were changed every day.

Eggs were round biconcave, olive green laid in clusters of 260-280 on the dorsal surface of tea leaves. They hatched in 6-7 days with 75% success. The post-embryonic development was for 39-46 days with six larval instars; first instars were light yellow, 2.81 mm in length, which grew 17-18 folds to reach brownish-black 6th instar stage, covered profusely with hair. Pictorial presentation of Life cycle of the insect is given in Fig. 5.27 with details in tabular form (Table 5.20).

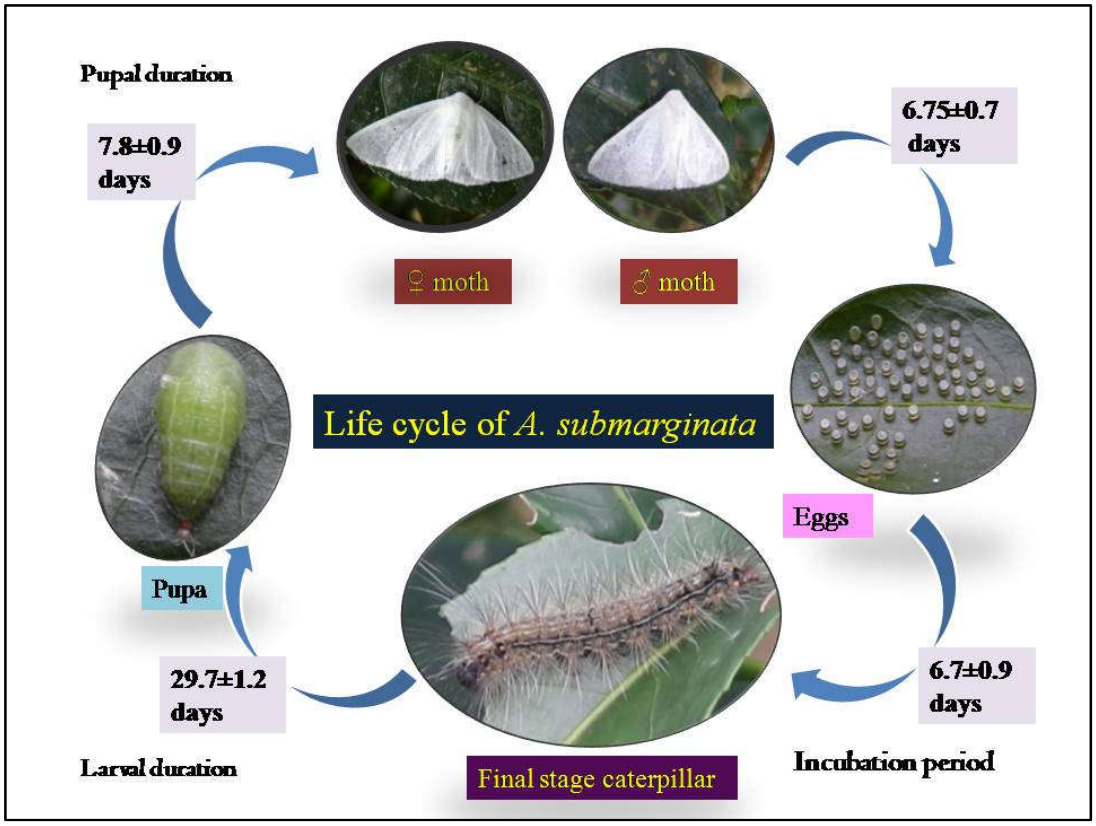


Fig. 5.27: Life cycle stages of *A.submarginata* reared in laboratory.

Table 5.20: Biological parameters of Life cycle of *A. submarginata* in laboratory.

	Observations *	
Biological parameters	Duration (days)	Average length (mm)
Egg Incubation	6.7±0.94 (n=30)	
1st larval Instar	8.5±0.57	2.81±0.31
2nd larval Instar	6.75±0.5	3.73±0.25
3rd larval Instar	5.5±0.57	9.6±0.96
4th larval Instar	5.75±0.5	17.7±0.82
5th larval Instar	3.5±0.57	34.8±0.77
6th larval Instar	2.8±0.78	39.1±0.99
Pupa	7.8±0.92	15.5±0.51
Adult longevity	6.75±0.73	
Secondary sex ratio (M:F)	1:0.5	
Fecundity	260-280	
Hatchability	75%	

*values are mean±S.D.

-experiments were conducted during November to February (average temp min: 13°C; max: 24°C; RH- 70±5%)

(ii) *Andraca bipunctata* Walker

Systematic position

Phylum: Arthropoda

Class: Insecta

Order: Lepidoptera

Family: Bombycidae

Genus: *Andraca*

Species: *A. bipunctata*

Moths of *A. bipunctata* were collected from the tea plantations of Darjeeling Terai and the Dooars. They were then allowed to mate and lay eggs in sterilized plastic container, towelled with tissue paper. Fertilized eggs were then transferred in fresh containers for hatching. After hatching the 1st instar caterpillars were transferred to small transparent plastic buckets. Thoroughly washed fresh tea leaves (with petioles of young leaves submerged in water filled 2ml micro centrifuge tube) were provided as food to the caterpillars. The bucket was sanitized and food was changed regularly. At 3rd instar stage the caterpillars were transferred to bigger transparent buckets provided with young and mature tea leaves as food. Leaves were changed as and when required. The eggs were light yellow, hard, leathery and oval with hatchability of 70%. The 1st instars were pale yellow, with black head capsule and distinct body segmentation, which later on grows up to 12-14 times when they reach maturity. Pictorial presentation of Life cycle of the insect is given in Fig. 5.28 with details in tabular form (Table 5.21).

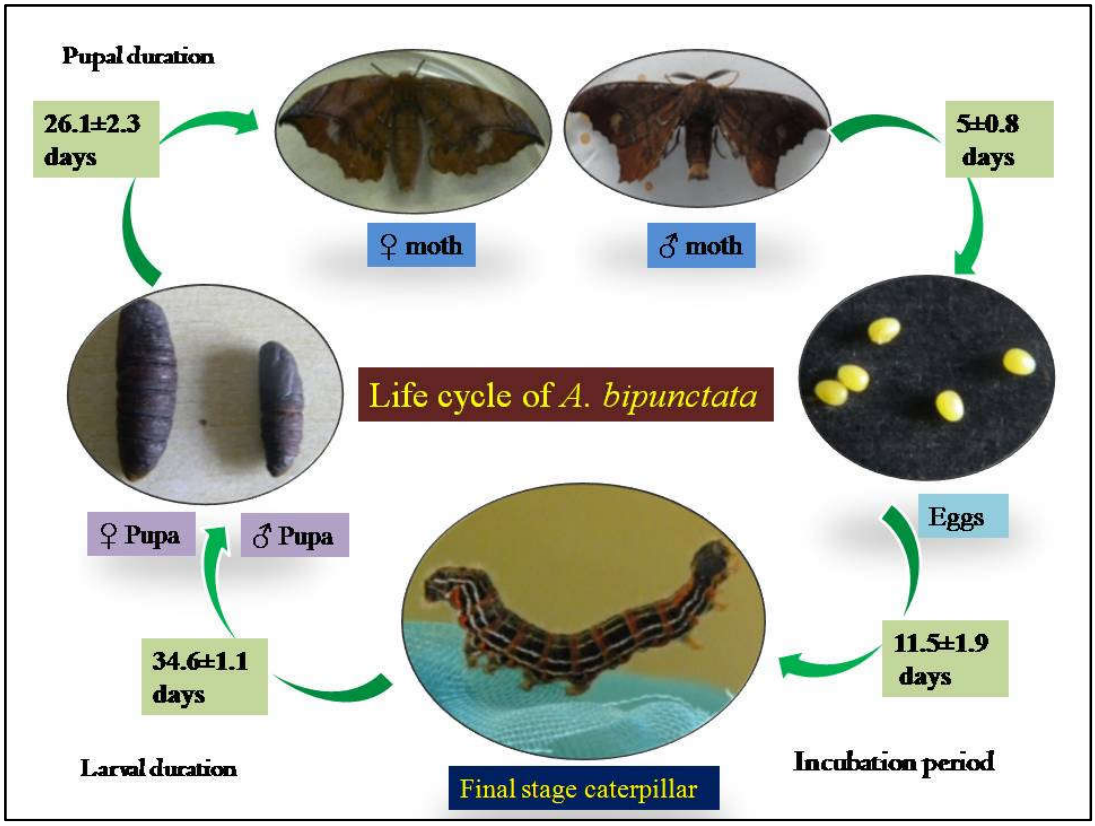


Fig. 5.28: Life cycle stages of *A.bipunctata* reared in laboratory.

Table 5.21: Biological parameters of Life cycle of *A. bipunctata* in laboratory.

	Observations *	
Biological parameters	Duration (days)	Average length (mm)
Egg Incubation	11.5±1.96	
1st larval Instar	8.2±0.95	4.12±0.88
2nd larval Instar	7.5±0.57	8.6±0.14
3rd larval Instar	6.5±0.57	14.6±0.15
4th larval Instar	7±0.81	21±0.10
5th larval Instar	6.7±0.5	48.1±0.32
Pupa(male)	26.1±2.37	18.8±0.1
Pupa(female)		25.4±0.11
Adult longevity♀	5.2±0.78	
Adult longevity♂	2.4±0.51	
Secondry sex ratio (M:F)	1:1	
Fecundity	360-400	
Hatchability	70%	

*values are mean±S.D.

-experiments were conducted during April to July (average temp min: 25°C; max: 32°C; RH-85±5%)

(iii) *Orgyia postica* Walker

Systematic position

Phylum: Arthropoda

Class: Insecta

Order: Lepidoptera

Family: Lymantriidae

Genus: *Orgyia*

Species: *O. postica*

Moths of *O.postica* were collected from the tea plantations of Darjeeling Terai and the Dooars. They were then allowed to mate and lay eggs in sterilized plastic container, towelled with tissue paper. Fertilized eggs were then transferred in fresh containers for hatching. After hatching the 1st instar caterpillars were transferred to small transparent plastic buckets. Thoroughly washed fresh tea leaves (with petioles of tea leaves submerged in water filled 2ml micro centrifuge tube) were provided as food to the caterpillars. The bucket was sanitized and food was changed regularly. At 3rd instar stage the caterpillars were transferred to bigger transparent buckets provided with mature tea leaves as food. Leaves were changed every day. The eggs were round, dull white in colour with hatchability of 73%. The larvae passed through six larval instars with a total post embryonic development period of 25-30 days. The 1st instar was about 2.3 mm, yellow in colour having patches of black bristles only on 4th segment. Post embryonic development lasted for 59-61 days with six larval instars. Pictorial presentation of Life cycle of the insect is given in Fig. 5.29 with details in tabular form (Table 5.22).

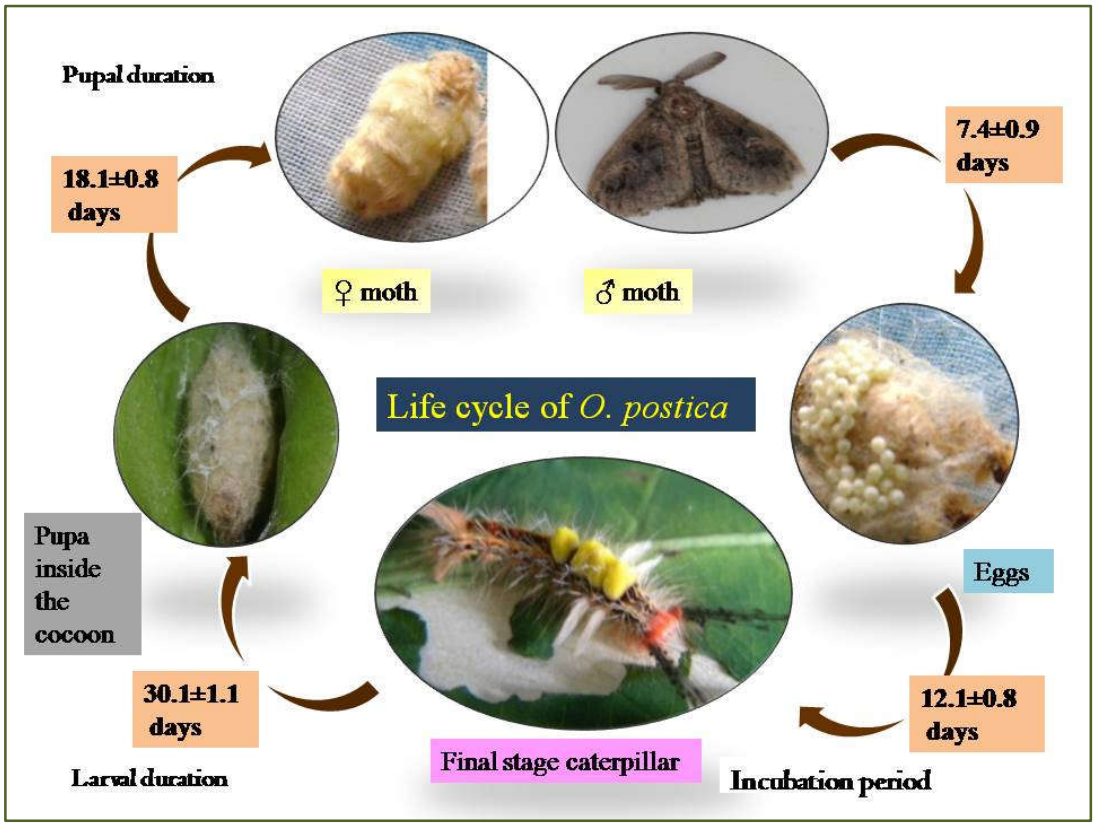


Fig. 5.29: Life cycle stages of *O.postica* reared in laboratory.

Table 5.22: Biological parameters of Life cycle of *O.postica* in laboratory.

	Observations *	
Biological parameters	Duration (days)	Average length (mm)
Egg Incubation	12.1±0.87	
1st larval Instar	8.7±0.78	2.3±0.15
2nd larval Insatar	4.18±0.75	6.27±0.18
3rd larval Instar	3.72±0.78	7.6±0.13
4th larval Instar	3.72±0.64	12.4±0.33
5th larval Instar	3.7±0.94	15±0.41
6th larval Instar	10.8±0.9	31.5±0.26
Pupa	18.1±0.87	14.1±0.78
Adult longevity	7.4±0.96	
Secondary sex ratio (M:F)	1:1	
Fecundity	250-300	
Hatchability	73%	

*values are mean±S.D.

-experiments were conducted during March to April (average temp min: 17°C; max: 27°C; RH-80±5%)

5.5.5.2 Rearing of the control insect (silk worm) in laboratory for testing safety of entomopathogens

***Bombyx mori* Linnaeus**

Systematic position

Phylum: Arthropoda

Class: Insecta

Order: Lepidoptera

Family: Bombycidae

Genus: *Bombyx*

Species: *B. mori*

Disease free eggs of *Bombyx mori* nistari (DFL) collected from local Central Sericulture farm, Matigara (Dist. Darjeeling) were kept in a water soaked cotton bed inside Petri plates and placed in an incubator at 24° C and > 70% humidity for hatching of the neonates. After hatching the paper containing the first instar was transferred to paper box and covered with fresh sterilized mulberry leaves. The caterpillars ate voraciouly and on finishing the layer of mulberry leaf, crawl to the upper layer of mulberry leaves. This upward movement of the caterpillars helped in the cleaning of the wastes and faecal matters which lay at the bottom. The caterpillars are provided with the fresh leaves 3-4 times a day and the amount depended on the advancement of the caterpillar stage. In about 21 days they were ready for pupation. The fifth (final) instar stopped feeding before spinning the silk cocoon. Adult moths emerged from the cocoons in about a week. Pictorial presentation of Life cycle of the silk moth is given in Fig. 5.30 with details in tabular form (Table 5.23).

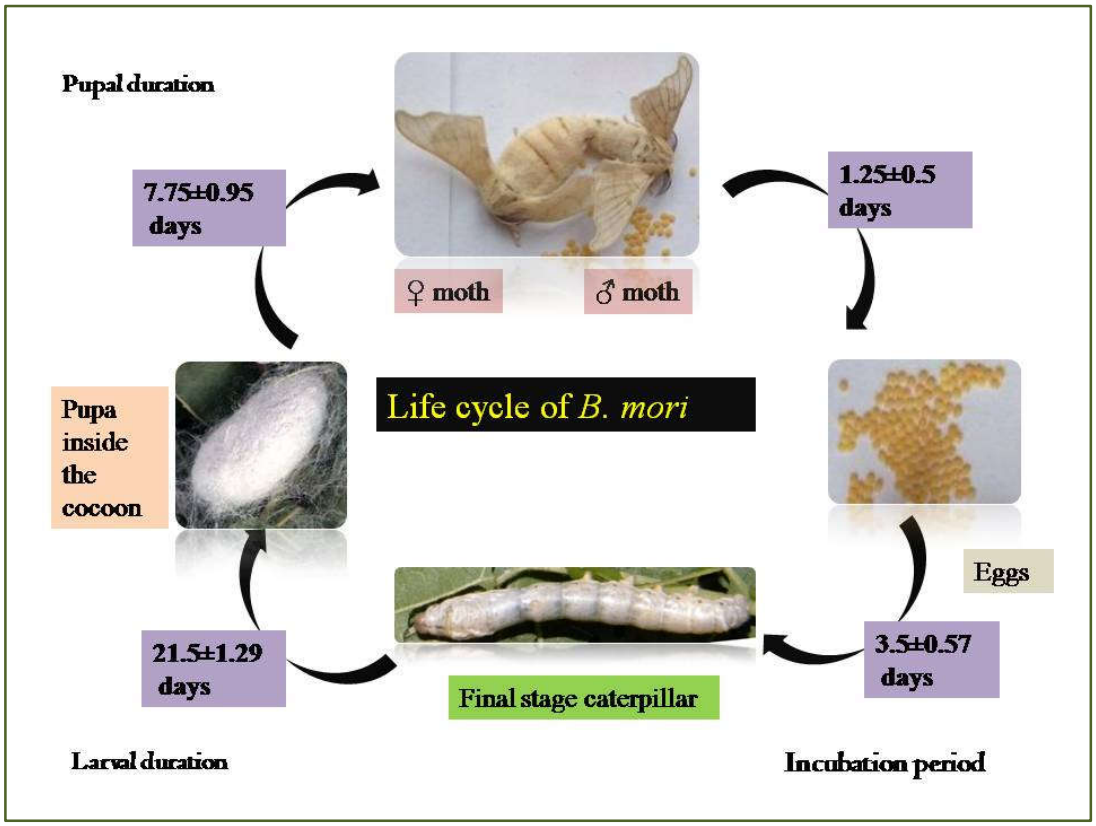


Fig. 5.30: Life cycle stages of silkworm (*B. mori* nistari) reared in laboratory.

Table 5.23: Biological parameters of Life cycle of *B. mori* in laboratory.

	*Observation
Biological parameters	Duration (days)
Egg Incubation	3.5±0.57
1st larval Instar	3.25±0.5
2nd larval Insatar	3.5±0.57
3rd larval Instar	3.75±0.5
4th larval Instar	5.5±1.2
5th larval Instar	7.25±0.95
Pupa	7.75±0.5
Adult longevity	1.25±0.5
Secondary sex ratio	1:1 (M:F)
Fecundity	350-400
Hatchability	95%

*values are mean±S.D.

-experiments were conducted in controlled temperature and relative humidity (24°C; RH-70%)

5.5.2 Bioassay (LT_{50} and LC_{50}) for *Bacillus* strains of *A. submarginata*

The percent mortality of second instar *A. submarginata* caterpillars when treated with *Bacillus* (Arc 01), ranged between 23% to 78% through 9 days observation. The LC_{50} value was recorded as 398.1 µg/ml with fiducial lower limit 353 µg/ml and upper limit 443.1 µg/ml. LT_{50} values were 7.28 days for 1000 µg/ml and 7.88 days for 750 µg/ml and 8.45 days for 500 µg/ml. In case of Arc02 strain of *Bacillus* the percent mortality of second instar larvae varied from 12% to 63% upto 9 days. The LC_{50} value was found to be 791.2 µg/ml with fiducial lower limit 662.5 µg/ml and upper limit 957µg/ml. The LT_{50} value was 5.5 days for 1000 µg/ml concentration. *Bacillus* sp. Arc03 showed a percent mortality between 26% to 79% upto 9 days. The LC_{50} was calculated as 342 µg/ml with fiducial lower and upper limit of 281.7 µg/ml and 414.9 µg/ml respectively. The LT_{50} of the same was found to be 5.28 days for 1000 µg/ml, 6.3 days for 750 µg/ml and 7.42 days for 500 µg/ml. *Btk* on the other hand showed the mortality ranging between 17% to 76% upto 9 days. LC_{50} value was 537 µg/ml with lower fiducial limit of 483.6 µg/ml and upper fiducial limit of 590.3 µg/ml. The LT_{50} values were 7.57 days and 8.5 days for 1000 µg/ml and 750 µg/ml, respectively (Fig. 5.31).

The data on dosage-mortality response of *A. submarginata* to Arc01, Arc02, Arc03 and *Btk* revealed that chi-square values were good fit of probit response. All the bioassays showed that there was no heterogeneity between observed and expected responses (Table 5.24).

Fig. 5.31: Bioassay of three entomopathogenic *Bacillus* strains: Arc01, Arc02, Arc03 and *Btk* (reference).

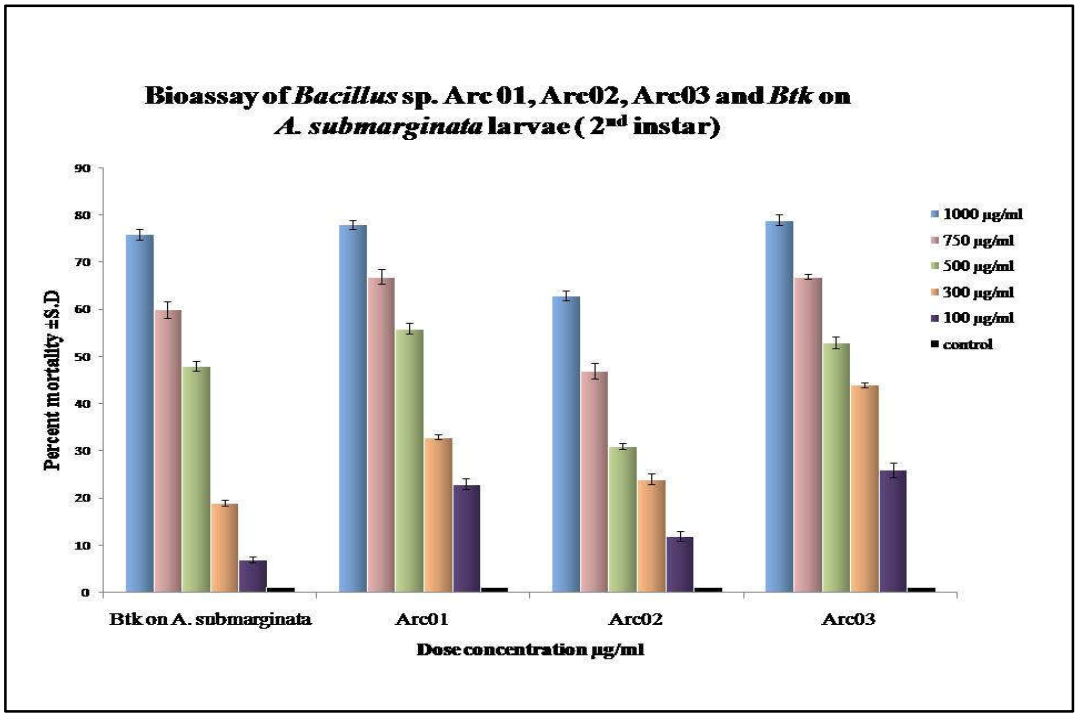


Table 5.24: Bioassay results of *Bacillus* strains: Arc01, Arc02, Arc03 and *Btk* (reference) on *A. submarginata* larvae (2nd instar; n=90).

						Fiducial limits		
Bacterial isolate	**#Concentration**	**% mortality**	**Heterogeneity (χ2)**	**Regression equation**	**LC_{50} (µg/ml)**	**Lower**	**Upper**	**LT_{50} (days)**
Arc 01	**1000** **750** **500** **300** **100**	**78** **67** **56** **33** **23**	**5.69112**	**Y=2.587569x+1.52924**	**398.1**	**353**	**443.1**	**7.28 for 1000** **7.88 for 750** **8.45 for 500**
Arc 02	**1000** **750** **500** **300** **100**	**63** **47** **31** **24** **12**	**4.81019**	**Y=2.901032x+1.47781**	**791.2**	**662.5**	**957**	**5.5 for 1000**
Arc 03	**1000** **750** **500** **300** **100**	**79** **67** **53** **44** **26**	**3.0022**	**Y=2.533x+ 1.365**	**342**	**281.7**	**414.9**	**5.28 for 1000** **6.3 for 750** **7.42 for 500**
Btk	**1000** **750** **500** **300** **100**	**76** **60** **48** **19** **17**	**5.07422**	**Y=1.520x+0.833**	**537**	**483.6**	**590.3**	**7.57 for 1000** **8.5 for 750**

 # Concentration in µg/ml

5.5.3 Bioassay (LT_{50} and LC_{50}) for *Bacillus* strains of *A. bipunctata*

The percent mortality of early 2nd instar *A. bipunctata* larvae increased from 20% to 55% within 9 days when treated with Ab01 strain of *Bacillus* in laboratory. The LC_{50} value for Ab01 was found to be 664 µg/ml with fiducial lower limit 519 µg/ml and upper limit 849.6 µg/ml. The LT_{50} values were found to be 7 days for 1000 µg/ml, 7.67 days for 750 µg/ml concentrations. In case of Ab02 strain of *Bacillus,* the percent mortality ranged from 13 % to 60% upto 9 days. The LC_{50} value was found to be 785.6 µg/ml with fiducial lower and upper limits 644.9 µg/ml and 956.9 µg/ml respectively. The LT_{50} value was 7.5 days for 1000 µg/ml. In Ab03 strain, the percent mortality of larvae varied from 13% to 57% upto 9 days in the laboratory condition. The LC_{50} value was found to be 783.2 µg/ml with fiducial lower limit 630.3 µg/ml and upper limit 973.3 µg/ml. The LT_{50} value was 7.5 days for 1000 µg/ml. The percent mortality of larvae in Ab04 strain of *Bacillus* varied from 27% to 87% upto 9 days. The LC_{50} value was found to be 385.8 µg/ml with fiducial lower limit 305.9 µg/ml and upper limit 486.6 µg/ml, while LT_{50} values were 5.83 days, 7.5 days and 7.67 days for 1000, 750 and 500 µg/ml concentrations, respectively. In case of *Btk*, the LC_{50} value was found to be 787.7 µg/ml with fiducial lower and upper limits 642.6 µg/ml and 965.5 µg/ml, respectively. The LT_{50} value recorded was 7.65 days for 1000 µg/ml (Fig. 5.32).

The data on dosage-mortality response of *A. bipunctata* against Ab01, Ab02, Ab03, Ab04 and *Btk* revealed that chi-square values were good fit of probit response. All the bioassays showed that there was no heterogeneity between observed and expected responses (Table 5.25).

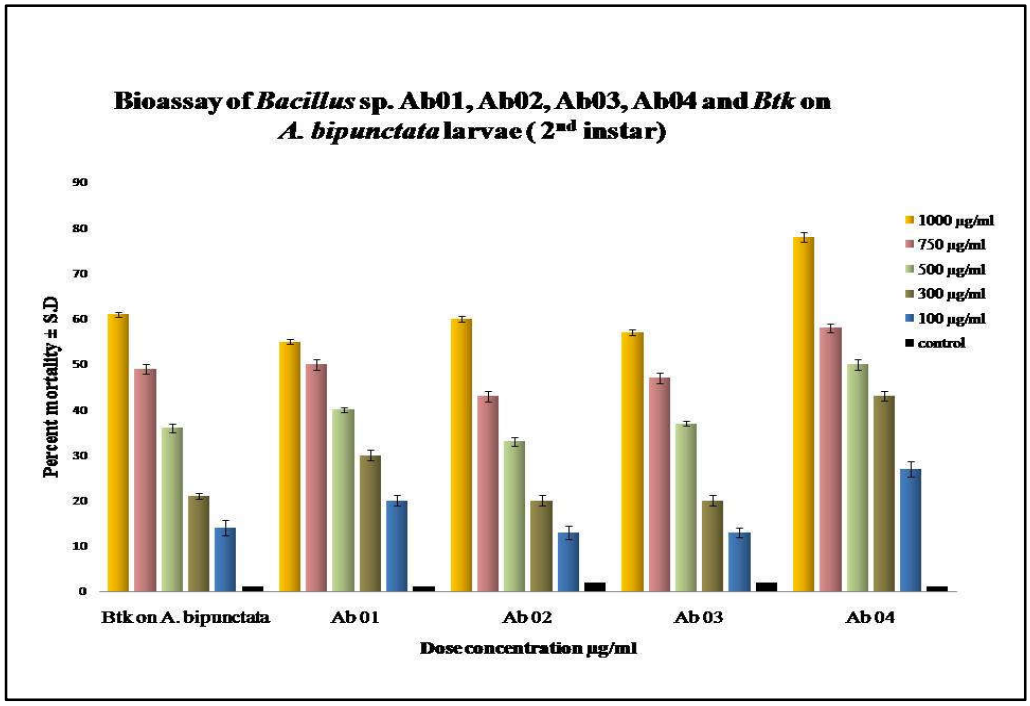


Fig. 5.32: Bioassay of four entomopathogenic *Bacillus* strains: Ab01, Ab02, Ab03, Ab04 and *Btk* (reference).

Table 5.25: Bioassay results of *Bacillus* strains: Ab01, Ab02, Ab03, Ab04 and *Btk* (reference) on *A. bipunctata* larvae (2nd instar; n=90).

						Fiducial limits		
Bacterial isolate	**# Concentration**	**% mortality**	**Heterogeneity (χ2)**	**Regression equation**	**LC_{50} (µg/ml)**	**Lower**	**Upper**	**LT_{50} (days)**
Ab01	**1000 750 500 300 100**	**55 50 40 30 20**	**2.03714**	**Y= 2.82220x+1.13888**	**664**	**519**	**849.6**	**7 for 1000 µg/ml 7.67 for 750 µg/ml**
Ab02	**1000 750 500 300 100**	**60 43 33 20 13**	**4.93654**	**Y= 2.09497x+1.38113**	**890.7**	**723.2**	**1096.9**	**7.5 for 1000 µg/ml**
Ab03	**1000 750 500 300 100**	**57 47 37 20 13**	**3.02716**	**Y= 2.92713x+1.38015**	**845.5**	**687.1**	**1040.4**	**7.5 for 1000 µg/ml**
Ab04	**1000 750 500 300 100**	**78 58 50 43 27**	**5.97441**	**Y= 2.58642x+1.19189**	**385.8**	**305.9**	**486.6**	**5.83 for 1000 µg/ml 7.5 for 750 µg/ml 7.67 for 500 µg/ml**
Btk	**1000 750 500 300 100**	**61 49 36 21 14**	**4.86453**	**Y= 2.89636x+1.34546**	**787.7**	**642.6**	**965.5**	**7.65 days for 1000 µg/ml**

 # Concentration in µg/ml.

5.5.4 Bioassay (LT_{50} and LC_{50}) for *Bacillus* strains of *O. postica*

Bioassays have been carried out on the early 2nd instar *Orgyia postica* larvae using two of the *Bacillus* isolates Org 2A and Org 6A. The result showed that in case of Org 2A strain of *Bacillus,* the percent mortality of larvae varied from 17% to 67% upto 9 days. The LC_{50} value was found to be 543.3 µg/ml with fiducial lower limit 477.6 µg/ml and upper limit 659.6 µg/ml. The LT_{50} values were 6 days for 1000 µg/ml and 6.5 days for 750 µg/ml concentrations. In case of Org 6A the percent mortality of larvae varied from 21% to 78% upto 9 days. The LC_{50} value was found to be 354.8 µg/ml with fiducial lower and upper limits 299 µg/ml and 421.1 µg/ml, respectively. The LT_{50} values were 5.5 days for 1000 µg/ml, 6 days for 750 µg/ml and 6.19 days for 500 µg/ml concentrations. When *Btk* was applied to the *Orgyia* larvae the percent mortality varied from 20% to 76% upto 9 days. The LC_{50} value was found to be 386.8 µg/ml with fiducial lower limit 326.7 µg/ml and upper limit 457.9 µg/ml. The LT_{50} values were 6 days for 1000 µg/ml, 6.5 days for 750 µg/ml and 7.15 days for 500 µg/ml concentrations (Fig. 5.33).

The data on dosage-mortality response of *O. postica* against Org2A, Org6A and *Btk* revealed that chi-square values were good fit of probit response. All the bioassays showed that there was no heterogeneity between observed and expected responses (Table 5.26).

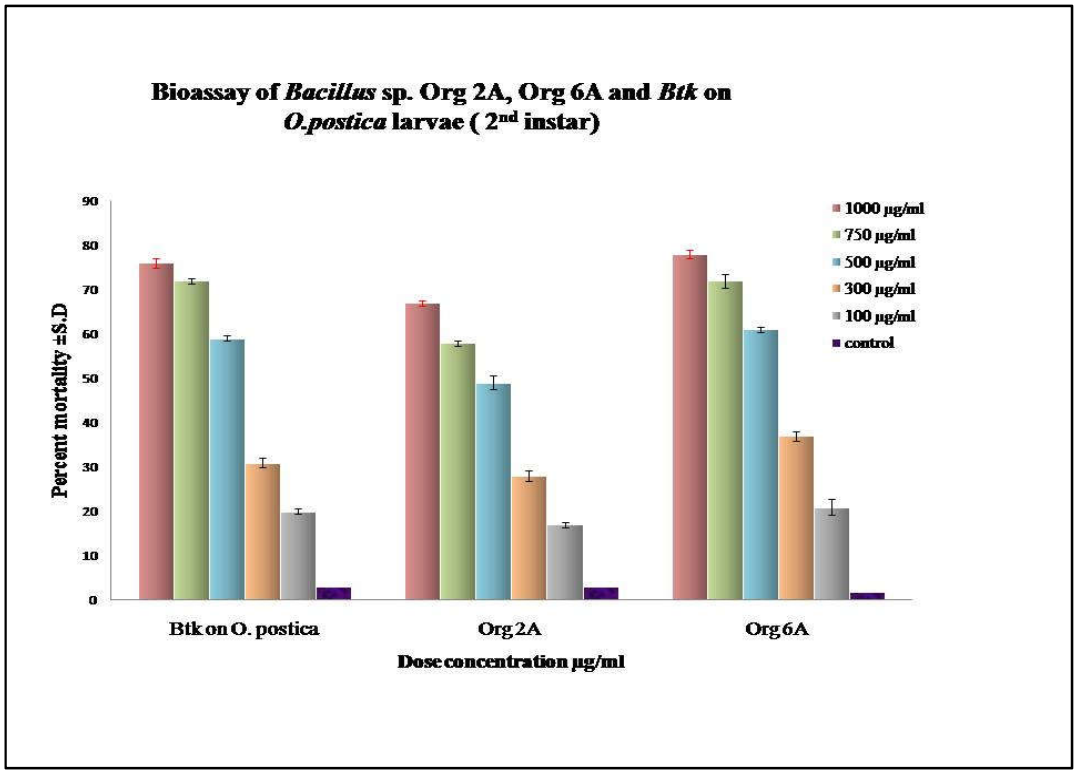


Fig. 5.33: Bioassay of two entomopathogenic *Bacillus* strains: Org2A, Org6A and *Btk* (reference).

Table 5.26: Bioassay results of *Bacillus* strains: Org 2A, Org 6A and *Btk* (reference) on *O. pstica* larvae (2nd instar; n=90).

						Fiducial limits		
Bacterial isolate	**# Concentration**	**% mortality**	**Heterogeneity (χ2)**	**Regression equation**	**LC_{50} (µg/ml)**	**Lower**	**Upper**	**LT_{50} (days)**
Org2A	**1000 750 500 300 100**	**67 58 49 28 17**	**2.72736**	**Y=2.735114x+1.4457**	**543.3**	**447.6**	**659.6**	**6 for 1000 µg/ml 6.5 for 750 µg/ml**
Org6A	**1000 750 500 300 100**	**78 72 61 37 21**	**2.88971**	**Y=2.55x+1.65871**	**354.8**	**299**	**421.1**	**5.5 for 1000 µg/ml 6 for 750 µg/ml 6.19 for 500 µg/ml**
Btk	**1000 750 500 300 100**	**76 72 59 31 20**	**6.50426**	**Y=2.58749x+1.68775**	**386.8**	**326.7**	**457.9**	**6 for 1000 µg/ml 6.5 for 750 µg/ml 7.15 for 500 µg/ml**

 # Concentration in µg/ml.

5.5.5 Cross-infection of the *Bacillus* isolates against silkworm larva

In the cross infectivity test using spore-crystal mixture of all of the nine isolates of *Bacillus* strains (Arc01, Arc02 and Arc03) isolated from *A. submarginata* cadavers, *Bacillus* strains Ab01, Ab02, Ab03 and Ab04) isolated from *A. bipunctata* cadavers, *Bacillus* strains (Org2A and Org6A) isolated from *O. postica* cadavers were prepared in five concentrations (1000, 750, 500, 300, 100 µg/ml) and the same were tested on *B. mori* (2^{nd} instar, n=90) by leaf dip method. Mortality were checked every 24 hrs upto 9 days. Bioassay setup showed that there was no significant mortality between inoculated and control set of silkworm larvae (Table 5.27, 5.29, 5.31) and there was no significant difference in the mortality when the mortality were compared through one way ANOVA (Table 5.28, 5.30, 5.32).

In general, no significant mortality could be observed in silkworms on exposure to the bacterial strains isolated from the sporadic lepidopteran tea pests under study. Suggesting these newly isolated *Bacillus* strains are safe for use in the tea gardens in North Bengal Terai area without harming the sericulture industry running parallaly in this area.

Table 5.27: Mortality in silkworm larvae when fed with bacteria isolated from *A. submarginata*.

		Arc01	Arc02	Arc03
Bacterial concentration (µg/ml)	Sample size x replicate =total	% mortality	% mortality	% mortality
1000	30x3=90	5.56	6.67	5.56
750	30x3=90	6.67	6.67	4.45
500	30x3=90	3.34	3.34	3.34
300	30x3=90	4.45	4.45	2.23
100	30x3=90	3.34	3.34	3.34
Control	30x3=90	3.34	3.34	3.34

Table 5.28: One way ANOVA comparing the effect of different *Bacillus* strains from *A. submarginata* (Arc01, Arc02 and Arc03) on *B. mori*.

Source	SS[§]	df	MS[¥]	
Between-treatments	0.345	2	0.1725	F =0.23596
Within-treatments	2.1931	3	0.731	
Total	2.5381	5		
F= 0.23596* P= 0.80321				

§ Sum of squares

¥ Mean of squares

*At 0.05 level, the means are NOT significantly different.

Table 5.29: Mortality in silkworm larvae when fed with bacteria isolated from *A. bipunctata*.

		Ab01	Ab02	Ab03	Ab04
Bacterial concentration (µg/ml)	Sample size x replicate =total	% mortality	% mortality	% mortality	% mortality
1000	30x3=90	11.12	10	7.78	10
750	30x3=90	10	8.89	10	11.12
500	30x3=90	7.78	8.89	10	10
300	30x3=90	8.89	7.78	8.89	8.89
100	30x3=90	6.67	6.67	7.78	8.89
Control	30x3=90	6.67	6.67	7.78	7.78

Table 5.30: One way ANOVA comparing the effect of different *Bacillus* strains from *A. bipunctata* (Ab01, Ab02, Ab03 and Ab04) on *B. mori*.

Source	SS[§]	df	MS[¥]	
Between-treatments	1.8248	3	0.6083	$F = 0.36524$
Within-treatments	6.6618	4	1.6654	
Total	8.4866	7		
F= 0.36524* P= 0.782887				

§ Sum of squares

¥ Mean of squares

*At 0.05 level, the means are NOT significantly different.

Table 5.31: Mortality in silkworm larvae when fed with bacteria isolated from *O. postica*.

		Org 2A	Org 6A
Bacterial concentration (µg/ml)	Sample size x replicate =total	% mortality	% mortality
1000	30x3=90	4.45	3.34
750	30x3=90	5.56	4.45
500	30x3=90	3.34	3.34
300	30x3=90	4.45	5.56
100	30x3=90	3.34	3.34
Control	30x3=90	4.45	5.56

Table 5.32: One way ANOVA comparing the effect of different *Bacillus* strains from *O. postica* (Org 2A and org 6A) on *B. mori*.

Source	SS[§]	df	MS[¥]	
Between-treatments	0.1971	1	0.1971	$F = 0.32$
Within-treatments	1.2321	2	0.6161	
Total	1.4292	3		
F= 0.32* P= 0.628609				

§ Sum of squares

¥ Mean of squares

*At 0.05 level, the means are NOT significantly different.

5.6 PCR amplification of 16S rRNA gene of most virulent strains

After isolation, purity of the total DNA of most virulent strains (Arc03, Ab04 and Org6A) was tested to make sure that the genomic DNA does not have any protein contaminations (Table 5.33). Next these pure DNA samples of most virulent strains isolated from three pest species was selected for PCR amplification of 16S rRNA gene using Universal Primer pair (Table 4.2). All the three strains under study and the reference *Btk* yielded a 1500 bp band after PCR (Fig. 5.34). All the PCR products were subjected to sequencing.

5.6.1 Characterization of the most virulent *Bacillus* strains using 16S rRNA

16S rRNA gene sequencing of the most virulent isolates, Arc03, Ab04 and Org6A yielded 1328, 1387 and 1386 nucleotides long amplicon with universal primer. The sequences were compared with the known sequences available in GeneBank of NCBI (National Centre for Biotechnology Information, http://www.ncbi.nih.gov/).

When sequences were analysed using BLAST search (Basic Local Alignment Search Tool) which provides a rapid comparison of related sequences, the 16S rDNA of Arc03, Ab04 and Org6A showed 99% sequence homology with *Bacillus thuringiensis* strain ATCC 10792 16S ribosomal RNA gene, partial sequence. [GenBank: NR_114581.1], *Bacillus thuringiensis* strain IAM 12077 16S ribosomal RNA gene, [GenBank: NR_043403.1] and *Bacillus thuringiensis* strain NBRC 101235 16S ribosomal RNA gene, [GenBank: NR_112780.1]. The partial sequences of these strains when aligned with related sequences (obtained from BLAST) using CLUSTAL W 2.0.12 program (multiple sequence alignment) resulted in the

generation of highest score of 99% among the sequences. These strongly supported that the isolated bacterial strains under study are indeed *B. thuringienseis.* The sequences of 16S ribosomal RNA genes were then submitted to NCBI and the same were given the Gene Bank Accession Numbers as KX245014, KX245015 and KX245016 for Arc03, Org6A and Ab04, respectively. Further, the sequence obtained for reference *Btk* showed difference with these virulent strains suggesting they are novel strains of *Bacillus thuringiensis* than the commercially available *Btk* which was used as reference for the study.

5.6.2 Cry gene amplifications of the most virulent strains

PCR analysis was carried out using the specific primers for the identification of *cry*1, *cry*2 and *cry*9 genes (toxic to lepidopterans) for most virulent *Bacillus* strains Arc03, Ab04 and Org6A and compared with reference *Btk* strain. Positive results were obtained for the *cry*1 (277 bp) and *cry*9 gene (354 bp) was obtained for Arc03, Ab04 and reference *Btk*. Further, Ab04 and reference *Btk* showed positive result for *cry*2 (1500 bp) gene whereas, Org6A only gave amplicon for *cry*2 gene (Fig. 5.35; 5.36; 5.37). The similar *cry* gene pattern obtained for the reference strain *Btk* indicates that these strains may belong to the serovar *kurstaki.*

Table 5.33: Quantification of genomic DNA of most virulent strains.

Strains	Wave length		Ratio[ж]
	$^{A}260$	$^{A}280$	
Arc03	0.024	0.12	2.0
Ab04	0.042	0.023	1.8
Org6A	0.017	0.009	1.9
Btk	0.019	0.01	1.9

[ж] The ratio lied between 1.7-2.0 indicating their purity.

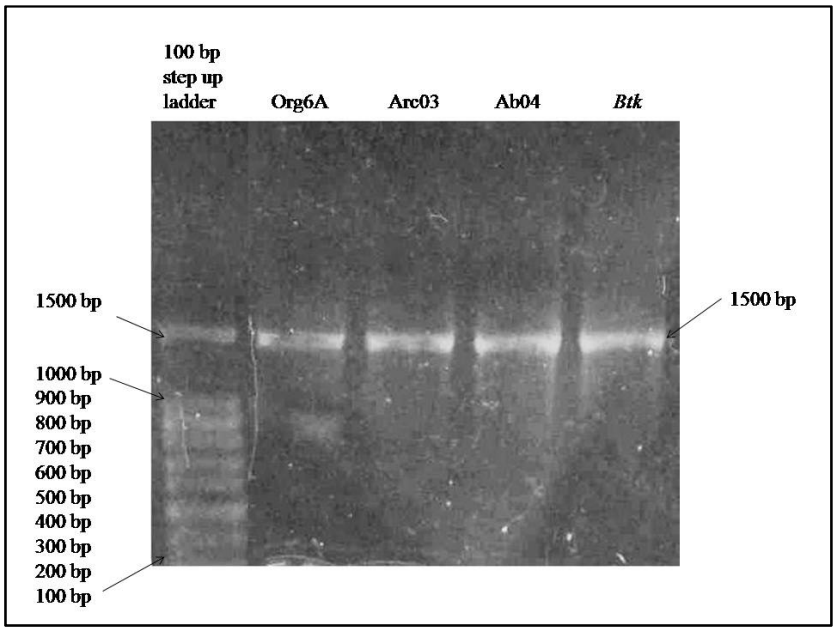


Fig. 5.34: 16S rRNA gene amplification of most virulent *Bacillus* strains (Arc03, Ab04 and Org6A) isolated from the pest species and reference strain *Btk*.

Nucleotide

GenBank

Bacillus thuringiensis strain ARC3 16S ribosomal RNA gene, partial sequence

GenBank: KX245014.1

FASTA Graphics

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  AUTHORS   Khewa Subba,S., Mukhopadhyay,A. and Bahadur,M.
  TITLE     16S rRNA partial sequence of Bacillus thuringiensis strain isolated
            from sporadic tea pest Arctornis submarginata (Lepidoptera:
            Lymantriidae) from Darjeeling foothills and plains
  JOURNAL   Unpublished
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  AUTHORS   Khewa Subba,S., Mukhopadhyay,A. and Bahadur,M.
  TITLE     Direct Submission
  JOURNAL   Submitted (13-MAY-2016) zoology, University of North Bengal,
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Nucleotide

GenBank

Bacillus thuringiensis strain ORG6A 16S ribosomal RNA gene, partial sequence

GenBank: KX245015.1

FASTA Graphics

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  TITLE     16S rRNA partial sequence of Bacillus thuringiensis strain Isolated
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  TITLE     Direct Submission
  JOURNAL   Submitted (13-MAY-2016) zoology, University of North Bengal,
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Nucleotide

GenBank

Bacillus thuringiensis strain AB04 16S ribosomal RNA gene, partial sequence

GenBank: KX245016.1

FASTA Graphics

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  TITLE     16S rRNA sequence of Bacillus thuringiensis isolated from sporadic
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  AUTHORS   Khewa Subba,S., Mukhopadhyay,A. and Bahadur,M.
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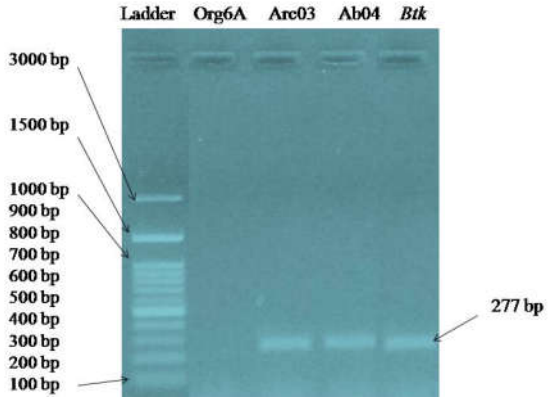

Fig. 5.35: Gene amplification of *cry* 1 of most virulent *Bacillus* strains (Arc03, Ab04, Org6A) isolated from the pest species and reference strain *Btk*.

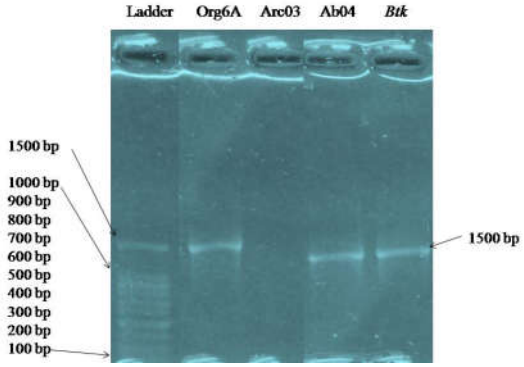

Fig. 5.36: Gene amplification of *cry* 2 of most virulent *Bacillus* strains (Arc03, Ab04, Org6A) isolated from the pest species and reference strain *Btk*.

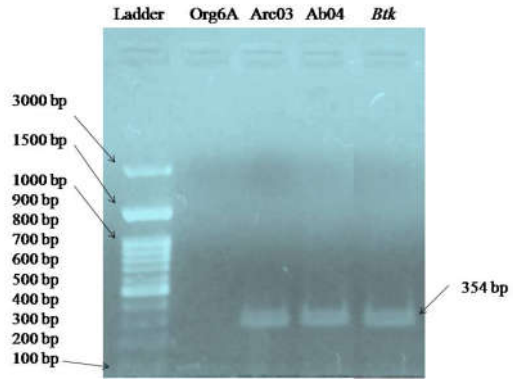

Fig. 5.37: Gene amplification of *cry* 9 of most virulent *Bacillus* strains (Arc03, Ab04, Org6A) isolated from the pest species and reference strain *Btk*.

6. DISCUSSIONS

Isolation of bacteria from three sporadic tea pests

Dead caterpillars of the lepidopteran tea pests, *A. submarginata, A. bipunctata* and *O. postica* after being collected from the natural populations of Sub Himalayan tea plantations were examined in the laboratory. The symptoms of bacterial infection were prominent (i.e. characteristically blackish with significant shrinkage of body and discolouration followed by rapid decomposition) which were similar to the observations of earlier authors (Shimanuki and Knox 1991, Lacey and Brooks 1997). When the healthy caterpillars were treated with the bacteria isolated from these cadavers, they developed same symptoms. The larvae dying of force killing as control did not show such symptoms after 12 hour incubation compared to the bacteria infected larvae after the same period. Typical symptom of death due to *B. thuringiensis* infection (larvae that died turned black, first around midgut and eventually throughout the entire larva) (Heimpel and Angus 1958) were evident in the treated larvae in the present study. Occurrence of naturally infected populations of the concerned pests in tea plantations possibly showed natural bacteriosis of lepidopteran insects that can lead to epizootics and mass mortality of natural populations (Osborn et al. 2002). The team of workers isolated and identified 29 bacterial strains from live, dead and experimentally infected *Hylesia metabus* (Lepidoptera: Saturniidae) larvae, and evaluated their pathogenic activity. The bacteria which caused mortality in the larvae were: *Pseudomonas aeruginosa* (60–93.3%), *Proteus vulgaris* (20%), *Alcaligenes faecalis*, *Planococcus* sp. and *Bacillus megaterium* (10%). All the bacteria-infected larvae showed typical symptoms i.e. they became flaccid, lethargic and stopped eating (Osborn et al. 2002).

The development of epizootic condition was studied in a dense population of larvae of the gypsy moth, *Lymantria dispar* (Lepidoptera: Erebidae). One of the two pathogens involved was a variant of *Streptococcus faecalis*. The feeding behaviour of the larvae enhanced the rate of larva-to-larva spread of the pathogens. Larvae in the first four instars fed most heavily in the tops of trees where dead larvae accumulated. These cadavers disintegrated and adhered firmly to the leaves, becoming an abundant source of inoculum for feeding larvae. Results from counts in the field and collections of larvae reared in the laboratory indicated that there was an increasing rate of infection and mortality that reached a climax when larvae were in the last instar. Observations indicated that the epizootic was host density-dependent and the rapid spread of pathogens in the susceptible population was enhanced by the feeding behaviour of the larvae during the early instars (Doane 1970). But it was found that the pathogen-host relationship became independent of host density when the pathogen threshold density is high and widely distributed. This situation occurs when pathogens are applied as microbial-control agents or after an extensive epizootic (Doane 1970). The fall armyworm *Spodoptera frugiperda* was susceptible to at least 20 species of entomogenous pathogens (bacteria). Some of these had the potential for a significant role in the management of the fall armyworm. Potential strategies included utilization of natural epizootics (Gardner et al. 1984).

Assaeedi et al. (2011) found that 60% of dead larvae of *Spodoptera littoralis* in Al-Jamoom area, an important entomofauna of economic importance in Saudi Arabia (Faraj-Allah and Al-Ghamdi 2003) harboured *Bacillus thuringiensis*. Numerous *Bt* subspecies have been isolated from dead or dying insect larvae and in most cases the isolate has toxic activity to the insect from which it was isolated (Goldberg and Margalit 1977, de Barjac 1981, Hansen et al. 1996, Nandish et al. 2011). These

organisms have a narrow host range in the orders Coleoptera, Diptera and Lepidoptera and can proliferate within the bodies of their host insects. When the infected insect larva dies, the dead insect carcass usually contains relatively large quantities of spores and crystals that may be released into the environment (Prasertphon et al. 1973, Grassi and Deseö 1984, Aly 1985). Growth of *Bacillus thrungiensis* in non-target organisms has also been described. Abika (1986) reported recycling of naturally occurring *Bt* in insect cadavers when competitive microorganisms were at a low density. Thus isolation of entomopathogenic bacteria from the host insect appears to be very effective, and promising for developing microbial pesticides.

Physiological characteristics of the bacterial isolates

Phenotypic characteristics of all the purified *Bacillus* strains isolated from *A. submarginata, A. bipunctata and O. postica* were rod shaped, endospore producing, gram positive and facultative anaerobes. Gram staining method can be used to tentatively identify and differentiate *B. thuringiensis* from morphologically indistinguishable yet different species (Obeidat et al. 2004). Morphology is affected by the medium on which the organism is grown and by temperature of incubation. Organisms are typical and in their most natural state in young cultures; in wet, unstained preparations, they are best observed by phase-contrast or dark- field microscopy. Such examination will show not only the shapes of organism but when prepared from suitable material, will show motility if present. Motility is a special feature of *Bacillus thuringiensis* (*Bt*) which distinguishes it from its close relative *B. anthracis* which are non-motile (Cowan and Steel 1993). The presently studied *Bacillus* strains showed typical characteristics of *Bt* in their cell morphology and crystal production during sporulation. Based on crystals, the distinguishing characteristic of *Bt* (Heimpel and Angus 1958), the isolates were identified as *Bt*

strains (Brussock and Currier 1990, Bai et al. 2002) . Patel et al. (2009) and Holt (1994) identified some isolates of *Bacillus* as *Bacillus thuringiensis* on the basis of morphological and biochemical criteria following Bergey's Manual of Determinative Bacteriology and other standard literatures. Entomopathogenic *Bacillus* from black fly (*Simulium* sp.) larvae and adults were also identified and characterized using Bergey's Manual (Cavados et al. 2001).

The crystal protein shape of the bacterial strains was found to be spherical in all the strains except Org2A and Org 6A which had oval crystals followed by Org1 which had a cubic crystal shape. On the other hand the shape of the crystal protein of *Bacillus thuringiensis kurstaki* (*Btk*) was bipyramidal. So, all the strains showed dissimilarity with *Btk* in respect of crystal protein shape. Such variation in crystal protein shape is known, where crystal proteins were different than common bipyramidal shape (Aronson et al. 1986, Lopez-Meza and Ibarra 1996, Ibarra et al. 2003, Choi et al. 2004, Kati et al. 2005, Kati et al. 2007). Zakeel et al. (2010) isolated rod shaped, spherical and rhomboidal shaped crystals. Lele and Nabar (2010) isolated 490 *Bt* strains from Sikkim having spherical and cubical crystals. According to Bernhard (1997), initial studies of *B. thuringiensis* isolates recorded in the literature reported that the bipyramidal crystals are more commonly obtained and are more toxic to lepidopteran larvae. However, the correlation between the crystal morphology and the level of insecticidal activity remains to be unclear (Bohorova et al. 1996). Keshavarzi (2008) found that most local *Bt* strains from different places of Iran, produced atypical crystals, often heterogenous in size and shape. Only a low percentage of the strains (17%) formed typical, bipyramidal crystals. Abundance of heterogenous crystals in *B. thuringiensis* strains has already been reported (Lecadet et al. 1999, Porcar et al. 1999) where more than 50% of *B. thuringiensis* strains produce

irregular or heterogenous crystals. The protein profiles of heterogenic crystals consist of many poorly defined components which could be a source of novel insecticidal properties (Juárez-Pérez et al. 1994, Burtseva et al. 1995, Chaufaux et al. 1997). The spore diameter in the present study was found to be larger than the control *Btk*, which indicates high diversity among the strains supported by the work of Shishir et al. (2014).

Bacterial growth rates during the phase of exponential growth, under standard nutritional conditions (culture medium, temperature, pH, etc.), define the bacterium's generation time (=doubling time). Generation times for bacteria vary from about 12 minutes to 24 hours or more. Generation time of all the *Bacillus* strains showed differences among themselves and also with that of *Btk*. As the generation time remains constant for any bacterial strain under standard nutritional conditions, differences in growth physiology can also be taken as an added phenotype for differentiating these *Bacillus* strains from one another and from *Btk*. The characterization of bacteria with the help of generation time is supported by the works of Kashyap and Amla (2007).

Viable spore count of the strains are also important to see the pathogenicity against target pest as toxicity of bacteria has been found to be directly proportional to the number of spores injested by that pest species (Heimple 1967). The cfu/ml of bacteria in present study corroborates with the above observation.

Biochemical characteristics of the *Bacillus* strains

Biochemical test is done with the purpose of comparison of the unknown with the known, the object being ability to say that the unknown is like A (one of the known bacteria) and unlike B-Z (all other known bacteria) (Cowan and Steel 2004). Moreover, the process helps in the initial identification of newly isolated strains.

A set of biochemical tests has been developed for the rapid identification of different biochemical types of *B. thuringiensis* isolates (Martin et al. 1985). This system is based on the biochemical tests that have been published for known varieties for which the serotypes have been identified (de Barjac 1981), and have been used for *B. thuringiensis* classification in many investigations (de Barjac and Frachon 1990, Doroszkiewicz and Lonc 1999, Lonc et al. 2001). Although this method in general does not make any distinction at a fine taxonomic level and does not exactly imply specific larvicidal activity, it may provide complementary information for more reliable identification and comparative studies (Święcicka and De Vos 2003).

Biochemical tests of the bacterial isolates included starch hydrolysis, casein hydrolysis and catalase tests. The reaction pattern showed that *Bt* was positive to all the tests, whereas some of the *Bacillus* isolates showed some variation/deviation from the *Btk*.

For further identification, API 20E kit was used. These tests, API 20E are standardized systems, associating 20 biochemical tests for the study of the carbohydrate metabolism of microorganisms. The API identification system is the one used for a rapid identification of bacterial strains, the method and system are well evaluated by several authors (Berkeley et al. 1984, Logan and Berkeley 1984).

In the present study API 20E was successfully used to characterize the bacterial isolates. This system of testing is also useful in comparing strains. However, as pointed out by Behrendt et al. (1999) and Peix et al. (2003), the identification of non-clinical isolates may often be wrong with these systems. There are few areas where species differentiation is difficult, for example four strains of *Bacillus* viz. *B. anthracis*, *B.cereus, B. mycoides* and *B. thuringiensis* are so closely related species that the only difference of *B. thuringiensis* being the presence of crystalline inclusions

which is absent in the former three (Cowan and Steel 2004). In fact (Gordon et al. 1973) regarded *B. anthracis* as a pathogenic variety of *B. cereus*, they also suggested that *B. mycoides* and *B. thuringiensis* should be treated as sub-species of *B. cereus* as they differed in very few characteristics which are all plasmid related and can be lost. Hence, identification of the entomopathogenic strains should be supported by other characters including molecular characterisation. The similarity matrix calculated using Dice matching coefficient, showed the differences in similarity levels in biochemical profiles of *Bt* and the different strains of *Bacillus*. This can give a rough idea about how similar two or more strains are based on biochemical profile. These results were agreed with those recorded by many workers (Schnepf et al. 1998).

The method of characterization of entomopathogenic bacteria with the help of morphological, physiological and biochemical tests was also evident in works of many other authors (Tyrell et al. 1981, Orduz et al. 1996, Lee et al. 2001, Bai et al. 2002, Choi et al. 2004). Characterization of newly isolated bacterial strains using biochemical tests indicated that the isolates were of *Bacillus* sp, with few variabilities.

Analysis of Crystal protein by SDS PAGE

Bacillus thuringiensis on sporulation produces crystal protein toxic to lepidopteran larvae when they ingest them (Dulmage et al. 1970, Miller et al. 1983). In the present study, the banding pattern of crystal protein in *Btk* (reference) varied from gel to gel. Some bands with lower molecular weight (56.6, 44.4, 29.5 kDa) were consistently observed with the appearance of new bands (128.4, 115.8, 110.7, 97, 75.1, 66 kDa) in different gels.

It was known that all serotypes of *B. thuringiensis* with one exception contains only one parasporal crystal protein of molecular weight of 145 kDa, 135 kDa or 130 kDa.

The serotype V on the other hand have two proteins of 135 and 130 kDa and sometimes a third protein of 65 kDa was also found. But contradictory results regarding both the number of polypeptides in the crystal and their molecular weights have been recorded by many workers in the past (Cooksey 1968, Sayles et al. 1970, Akune et al. 1971, Herbert et al. 1971, Glatron and Rapoport 1972, Bulla et al. 1977, Nagamatsu et al. 1978). Chestukhina et al. (1977) found that in addition to the three bands of *B. thuringiensis* var *galleriae* new bands corresponding to 110-65 kDa appeared when the crystal solution was kept between 4-20 °C. Stepwise degradation of 135-130 kDa could also be seen at 37°C, where 110 kDa and lower molecular weight bands were seen for the same strain. In case of the *B. thuringiensis* var. *insectus* too, three bands of 145 kDa, 95 kDa and 75 kDa changed to 145 kDa, 110 kDa, 100 kDa, 90 kDa and 75 kDa with deletion of 95 kDa band and addition of 110 kDa, 100 kDa and 90 kDa bands when pH of solubilizing buffer changed from 12.5 to 8.5. These results indicated that *B. thuringiensis* crystals contain endogeneous proteinase(s) capable of hydrolyzing crystal proteins into smaller fragments (Chestukhina et al. 1980). By end of 20th century the reported number of molecular weights of Lepidoptera-specific *B. thuringiensis* crystal proteins were found to be ranging from several thousands to well over 200,000 (Yamamoto 2001).

The extent of the proteolysis of crystal protein strongly depends on a number of factors: i.e. the content of the proteinases present in the crystal formed, the conditions used for the protein solubilization, the stability of the proteinases against the denaturing agents, etc. therefore incomplete elimination of proteinases on crystal-forming proteins may not produce reproducible data on their composition and molecular weight in spite of being the same strain. Therefore, the non-consistent bands of the reference *Btk* in the present study may be explained by the above study.

The crystalline inclusion dissolves in the gut of susceptible larvae and release one or more insecticidal proteins (endotoxins) of 27-140 kDa range (Charnley 1991). The SDS-PAGE analysis of the isolated *Bacillus* strains showed presence of several major polypeptides. The profile of crystal protein in SDS-PAGE is useful for characterization of delta-endotoxin families (Cavados et al. 2001). Shishir et al. (2012b) found that the thirteen of their active isolates revealed numerous protein bands with molecular weight ranging from 19kDa to 195kDa. But the most prominent band present in the isolates were that of ~60kDa, ~130 kDa and ~135kDa which indicated the presence of Cry2, Cry1 and Cry9 proteins (Crickmore et al. 1998). But it was found that 130kDa protein band can encode *cry*1, c*ry*4 and c*ry*9 gene (Shevelev et al. 1998). Kitnamorti et al. (2011) found great variations in cry protein ranging from 25 kDa to 60 kDa. In similar study, Valicente and Lana (2010) found different protein profile, with molecular mass ranging from 30 kDa to 205 kDa.

In the present study *Btk*, Arc01, Arc03 and Ab04 showed 128, 128.4, 122.7 and 129.8 kDa bands, respectively, they were the size of ~130 kDa band corresponding to cry1 toxin. Similarly, the results showed that Arc01 had 64 kDa, Ab01 had 56.7 kDa, Ab03 had 56.2 kDa, Ab04 had 56.5 kDa and Org6A had 57.7 kDa band, all correspond to ~60 kDa of Cry2 toxin. Two crystal proteins with molecular masses of ~65kDa and 130kDa was found in the Bn1 strain by Kati et al. (2007). 130kDa was confirmed by the PCR for Cry1 toxin (Honigman et al. 1986). Yilmaz et al. (2013) in their study revealed that the local isolates of *Bt* from warehouse of Turkey produced several protein bands with molecular size ranging from 28 to 130kDa. Similarly, Opondo et al. (2010) reported three major protein bands of 28, 65 and 130kDa in their isolates. HD-1 and many other isolates have been shown to produce multiple

protein bands (Calabrese et al. 1980). However, Morris et al. (1998) reported two major bands of 68 kDa and 130 kDa in the standard *Bt* HD-1 strain.

Patel and Ingle (2012) isolated three native strains of *Bt* and found that one of the strains GS4 produced multiple bands of proteins including 88 and 54 kDa as reported earlier by Lopez-Meza and Ibarra (1996). It also showed presence of other bands of size 175, 135 and 97 kDa indicating presence of more than one Cry proteins. In a study, Crickmore et al. (1998) reported prominent bands of 130 and 66 kDa corresponding to Cry1 proteins in the isolate GN24 and 60 kDa characteristic of Cry2 proteins in the isolate UP1 along with faint bands of 85 and 40 kDa. Presence of large number of crystal proteins in a single isolate makes it a versatile insect toxicant having a wide range of entomopathogenicity (Krywienczyk et al. 1978). Tohidi (2013) showed the presence of 90-100 kDa bands in *Bt* strain IBL200. Lopez-Meza and Ibarra (1996) showed that novel strain of *Bt* LBIT-113 produced two peculiar crystal proteins with molecular size 88 and 54 kDa. They did not correspond to any of the typical cry protein. But 88kDa protein on trypsin digestion produced a 60kDa fragment. In the present study, 85.8 kDa band in Arc02 and 88.9 kDa band in Ab02 were observed which corroborates the above results. However, *Bt* subspecies *cameron* appeared be close to this strain as it has the crystal protein of 88.1, 81.2, 54 and 53 kDa. Shauka et al. (2010) and Chilcott and Ellar (1988) too found 130kDa and 65kDa bands in the *Bt* isolate which corresponded to cry1 and cry 2 toxin gene. Maeda et al. (2000) isolated *Bt* (isolate F735) which produced major bands of 125, 116, 100 and 65 kDa, whereas, another isolate (F747) produced a major band of 50 kDa. In the present study Arc02 and *Btk* had 117.3 kDa and 115.8 kDa bands which are almost similar to 116 kDa reported by Maeda et al. (2000). Orduz et al. (1994) analysed crystals of Columbian strains of *Bt* by SDS PAGE. They reported presence of 135 to

144 kDa bands and a single band of 67 kDa in two of their isolates. In another strain they reported the presence of 100, 80, 75, 67, 65, 40, 30 and 28 kDa bands. Similar results were found in the present study. All the strains showed consistently a band of ~40 kDa and additionally a band of ~ 30 kDa was also observed in Ab03 and Ab04 including *Btk*. However, Armstrong (1985) found 27kDa protein band which corresponded to a cytolytic toxin. Zhu et al. (2009) found that 80 locally isolated strains showed six different protein profiles having bands from 40 to 130kDa but none of them gave any PCR product when assayed with all the known primers. These protein profile suggests that these *Bt* strains may harbour novel *cry* genes.

Qualitative and Quantitative assay of whole cellular proteins of the isolates

The usefulness of whole cell SDS-PAGE as a sensitive tool for providing valuable information on distinguishing bacteria in species and subspecies level, has been shown repeatedly (Costas 1992, Berber 2004). The difference in the banding pattern of the bacteria indicates that they may be different isolates.

Therefore to see the difference in the whole cell protein of the *B. thuringiensis* isolates SDS PAGE was used. Electrophoresis of whole-cell proteins produced patterns composed of 2-8 bands with molecular weights of 10–126.6 kDa. The difference in banding pattern among the isolates and reference strain *Btk* indicated heterogeneity of the *B. thuringiensis* strains and allowed discrimination of the isolates. The protein profile of the isolates could be distinguished into three groups on the basis of molecular weight of the corresponding bands and it was seen that all the isolates had bands in Group I (25 -59 kDa) rather than group II or III. The differences occurred in the later groups. Our results corroborate the finding of Haggag and

Yousef (2010). In similar study of the whole cell protein of lepidopteran specific *Bt*, an array of 10 to 21 bands of molecular weight 18 to 140 kDa were observed by Patel et al. (2009). When whole cell protein of nine *Bt* strains was analyzed, Haggag and Yousef (2010) found presence of 8-17 bands with molecular mass ranging from 28 to 254 kDa. It is reported that these difference in proteins are responsible for distinction of strains (Fu et al. 2008, Barth and Stiles 2010). The whole cell protein profile has been useful in determination of similarity between the bacterial isolates (Costas 1992, Święcicka and De Vos 2003). According to Berber (2004), the protein profile of whole cell can differentiate the *Bacillus* up to species level and sometimes up to subspecies level. Allwin et al. (2007) reported diversity of the native *Bt* isolates in Tamil Nadu. Similarly, Berber and Yenidünya (2005) have shown diversity of the native alkaliphilic *Bacillus* spp. in Turkey. Fakruddin et al. (2012) detected 10 to 150 kDa proteins in native *Bt* strains of Bangladesh, whereas the reference *B. thuringiensis* HD-73 used in the study showed 60 to 140 kDa protein bands. Syed Arshi et al. (2012) carried out whole cell protein analysis, using SDS-PAGE and categorized 60 isolates into 16 groups (S1-S16), on the basis of banding patterns. Our results of the whole cell protein analysis of *Bacillus* isolates showing protein bands ranging from showed 10 to 126.6 kDa (Arc01, Arc02 and Arc03) 29 to 110 kDa (Ab01, Ab02, Ab03 and Ab04) and 30.8 to 78.5 kDa (Org2A and Org6A) are in concurrence with the above findings. Whole cell banding pattern variability has been used to construct a dendogram, and compare with the known strain to identify the genus of the isolates (Adwan and Adwan 2004, Patel et al. 2009). Haggag and Yousef (2010) suggested that the difference in the whole cell protein content among different *Bt* isolates indicated that they are distinct strains.

Plasmid profiling of the bacterial strains

It has been reported that *Bacillus thuringiensis* commonly harbours a number of large plasmids with different molecular mass. In a study, Shishir et al. (2012a) have found plasmids that ranged from 10-15 kb with *Btk* HD-73 having 12 kb band. In another study, Shishir et al. (2014), found clearly visible plasmids ranging from 3kb to more than 16kb. 81% of the visible plasmid bands fall into the range of 10-13 kb. Further, Shishir et al. (2012a), observed that plasmid bands ranged from 15 kb to well up to 22 kb, but 15 kb band was evident in all isolates of *Bt* strains. Similarly, Apaydin et al. (2005) reported presence of a major plasmid band of 15kb in 33 *Bt* isolates. In addition they also found plasmids of varying size between 15 kb and 22 kb in *Btk* from Korean soil. In present study too, the size of plasmids ranged from 16 kb (Ab01) to 20.7 kb (Arc01) which is in agreement with the above results. Lee et al. (2001) found that their *Bt* strain (K2) had different plasmid pattern than *Bt kurstaki* HD-1, which has a typically small plasmid. In a similar study, Yilmaz et al. (2013) found 19.3 kb plasmid band comparable to 19.4, 19.8 and 19 kb plasmid bands of Ab04, org 6A and reference *Btk,* respectively, in the present study.

The number of plasmids in *Bt* strains may vary from 1-17 (González 1981, Aptosoglou et al. 1996). The size, number and molecular mass of the plasmids suggest genetic divergence between the strains of that species. Plasmid patterns are used to compare and characterize the strains frequently (Ibarra and Federici 1986, Ibarra et al. 2003).

In general plasmids of each isolate showed similarity manifesting almost same molecular weight ~19 kb as has been documented earlier by Adang et al (1991) and Uozumi et al. (1977). Bozlağan et al. (2010) compared plasmids of 17 *Bt* isolates and

the standard strain *Btk* and have found that all the isolates exhibited approximately 19 kb plasmid bands as has been found true in the present study.

The size of the plasmids in *Bt* may vary from 2 to 1000 kb with most *cry* toxin genes being situated on these plasmids (Carlson and Kolstø 1993, Opondo et al. 2010). However, the earlier reports have shown that the genes encoding crystal proteins may be located on the chromosomes as well as on a plasmid in the subspecies *kurstaki* (Held et al. 1982) and subspecies *thuringiensis* (Klier et al. 1982). *Bt* plasmids are studied either to locate *cry* genes (Gonzalez and Carlton, 1980, Kronstad and Whiteley, 1983) or transfer between different strains (González et al. 1982, Aronson et al. 1986), but they are also used to characterize the bacterial strains (Aptosoglou et al. 1996, Porcar et al. 1999, Vilas-Bôas and Lemos 2004) by comparing with standard strains (Padua et al. 1984, Ibarra and Federici 1986, Ibarra et al. 2003). Presence of different plasmid profiles among the isolates belonging to the same strain(Aptosoglou et al. 1996), indicates heterogeneity of the *B. thuringiensis* isolates.

Many workers have compared plasmid patterns to distinguish isolates even within same 'serovar' (González 1981, Benintende et al. 2000). Therefore, the patterns of plasmids isolated from tea pests in the present study are comparable to the reference strain *Btk* as also has been established by many earlier works, for other bacillae.

Bioassay (LT_{50} and LC_{50}) for *Bacillus* strains

Mass and laboratory rearing of pests to know the basic biology of a pest is a key component of IPM strategies (Sorensen et al. 2012). Also, it is very important to have same stage larvae in huge quantity for bioassay (Ahmad et al. 2008). Populations of

the pests were reared in the laboratory for one generation to obtain sufficient numbers of insect to perform bioassay.

The infectivity of the bacterial strains (Arc01, Arc02, Arc03, Ab01, Ab02, Ab03, Ab04, Org2A and Org6A) was determined through bioassay. Before going for field study it is necessary to determine the toxicity of the entomopathogen in laboratory condition (Monnerat et al. 2007). The test conducted in laboratory conditions in case of spore and crystal producing bacteria with purified crystals may not be actual representation of the toxicity of a strain under natural conditions. So, bioassays with spore-crystal mixtures are desirable, therefore conducted both under laboratory and field conditions and compared with the activity of commercialized strains such as *Btk* to determine the exact potential of the strain (Itoua-Apoyolo et al. 1995, Saadaoui et al. 2009).

In some insect hosts, after lower sub lethal dosages of crystal, the high pH of the gut rapidly falls, which allows spores to germinate in the gut and the resulting bacteria to increase in numbers. When the body contents can support no more bacteria, spores are formed, allowing the spores to get released by disintegrating and surviving in the environment until further healthy larvae are infected (Burges 2001). In bioassay tests, the spore–crystal mixture has a higher larvicidal activity as had been reported earlier (Johnson and McGaughey 1996, Johnson et al. 1998, Yaman et al. 1999, Mohan and Gujar 2001). They established that the addition of spores to delta-endotoxin was essential to induce significant mortality in larvae of myiasis-causing dipteran *Chrysomya albiceps* (Wiedemann).

In the present study, leaf dip method was used in the bioassay (Pandey et al. 2009). Leaf disk bioassay tend to be more reliable delivery system than diet incorporation

methods, as they have the advantage that they mimic natural conditions, avoiding problems with the sporulation of *Bt* spores in artificial diets, and permit a natural feeding behaviour of the test insects (Navon 2000, Martínez et al. 2004). Using early instars for bioassay is advantageous as third instar larva of Lepidoptera are less susceptible to the *Bt* than younger caterpillars (Navon et al., 1990).

In all the cases it was found that the LC_{50} value of Arc01, Arc02, Arc03, Ab01, Ab02, Ab03, Ab04, Org2A and Org6A were comparatively lower than the commercially used biopesticide, i.e. *Btk* for lepidopteran caterpillars. Both low LC_{50} and LT_{50} values proclaimed a higher toxicity of the newly found strains in question. When bioassay was done using neonate larvae of *Epinotia aporema*, Lopez-Meza and Ibarra (1996) found that their isolate INTA TA24-6 had almost three fold higher insecticidal activity compared to HD-73 strain of *Btk*, but had lower activity than HD-1. Although the LC_{50} value was slightly higher than *Btk* in case of Arc02 and Org2A strains yet the LT_{50} values were lower or equal to *Btk*. The low LC_{50} and LT_{50} values of the new strain of *Bacillus* makes it a promising potential entomopathogenic bacterium that can be further developed in to biopesticide with proper formulation and field testing. Sattar et al. (2008) and Netravathi et al. (2009) explained that the variations in efficacy against different pests may be due to varying number of *cry* genes and the absence of specific binding sites. These results suggest that the *Bacillus* strains under study may be considered for further exploration. It has been found that the estimated LT_{50} value decrease with the increase in dose (Trang and Chaudhari 2002). LT_{50} values provide additional information that the pathogen that kills quickly will help reduce the damage by the concerned pest (Kadir et al. 1999). It has also been found that LT_{50} value increases with increase in doses used in bioassay (Van Beek et al. 1988). It has been found that two insecticides with similar LC_{50} but one with low LT_{50}

value is more effective as it requires less time to kill the insect (Ahmad et al. 2005). Natural bacterial isolates have been found to be of higher activity than standard stock of *Btk* (Patel et al. 2009) The activity of strains isolated from different insect species have also been found to be higher insecticidal potential than that of reference strain (Bai et al. 2002, Kati et al. 2007).

It was also noted that the larval developmental period get delayed after a larva gets infected with sub lethal dosage of bacteria (Fast and Régnière 1984, Bauce et al. 2002, Moreau and Bauce 2003). The larvae in lower dosages (100 and 300µg/ml) showed reduced length, size and weight as also has been observed in the present study. It was in agreement with the data based on a dose of *Bt* that killed 40% *Plutella xylostella* larvae, where the phenomenon of reduced growth was evident (Grbin 1997). Schoenmaker et al. (2001) also observed that larval developmental period gets prolonged in spruce budworm larvae after treatment with *Btk*. Similar observations were available for *Helicoverpa armigera* after being sprayed with *Btk* formulation (Mohan and Gujar 2001).

Therefore, the result suggests that the bacterial strains studied have the potential to be developed as microbial biopesticides in future with parallel effects as *Btk*, which is already in use for controlling different lepidopteran tea pests.

Cross-infection of the *Bacillus* isolates against silkworm larva

The *Bacilllus* strains Arc01, Arc02, Arc03 from *A. submarginata*, Ab01, Ab02, Ab03 and Ab04 from *A. bipunctata*, Org 2A and Org 6A from *O. postica* were found to be pathogenic to their host species but before formulating these entomopathogenic bacillae and go for their application through field spray it is advisable to determine

their toxicities to beneficial lepidopteran insects which might be at risk after wide application in tea plantation to suppress pest. As the silk worm industry is running parallel with tea industry in northern region of West Bengal, the cross-infectivity of the isolated *Bacillus* strains to silk worm was tested. Status and potential of *Bt* insecticide in a tea-silkworm ecosystem has been reviwed extensively by Dashora et al. (2017) and encouraged the use of local strains of bacillae with novel toxins. Invertebrate animals offer alternative, less expensive animal models to study host-microbe interactions. The larvae of silkworm *Bombyx mori* are useful animal model to study infections by bacteria or fungi that are pathogenic (Hossain et al. 2006). Early second instar larvae of multivoltine silk worms normally reared in North Bengal Terai region were taken for the experiment. The Japan Plant Protection Association in 1973 proposed a bioassay method with silk worm, *Bombyx mori* for the quality control of all *Bt* formulations commercially produced in that country (Asano and Miyamoto 2004). Various strains of *Bacillus thuringiensis* may have high killing potentiality against looper pest of tea, however before their application as microbial pesticide the harmful effect of these bacterial strains on silk worm needs confirmation (Mukherjee and Singh 1993b).

In the present study it was found that the all the strains of bacteria isolated from *A. submarginata, A. bipunctata* and *O. postica* were not infecting the silk worm larvae, leading to their significant mortality. So, from these experiments it can be inferred that the newly isolated bacterial strains, Arc01, Arc02, Arc03, Ab01, Ab02, Ab03, Ab04, Org 2A and Org 6A with proper formulation are apparently safe for spraying in the tea plantation for controlling the respective pest species. Any consequential damage to beneficial insects specially silk worm industry in particular is ruled out. Also, from literature it was evident that already used biopesticides especially *Bt* are

largely harmless to beneficial insects such as honey bee, silk worm and mammals including human being (Bajwa and Kogan 2001). *Bacillus thuringiensis kurstaki* that are effective against lepidopteran pests, are relatively harmless to silk worms (Khetan 2000). Concern over potential harm to silk worm industry has led some countries to prohibit the use of *Bt* product, a position that now might logically be reviwed given the diversity of available *Bt* strain. Therefore, it can be concluded that the bacterial isolates from the lepidopteran tea pests under study are not harmful to silkworms and silk industry atlarge and therefore, can be further developed as biopesticides for future use.

PCR amplification of 16S rRNA gene of most virulent strains and *Cry* gene amplifications of the same

As the members of *Btk* group are virtually hard to distinguish just on the basis of phenotypic criteria alone (Woese 1987), therefore, the study of 16S rDNA sequence is necessary to confirm the result (Ash et al. 1991, Bravo et al. 1998). It has been considered as one of the important polyphasic approach for bacterial classification (Clarridge, 2004). 16S rRNA sequencing has been suggested to be useful tool in the discrimination between the species in the *Bacillus* cereus group (Mohamed et al., 2006). Assaeedi et al. (2011) using 16S rRNA sequencing of PCR products confirmed that the eight environment-derived isolates that formed parasporal inclusions and exhibited strong activity against Lepidoptera were novel isolates of *Bacillus thuringiensis*.The universal primer has been used to amplify 16S rDNA sequence (Kati et al. 2007, Shishir et al. 2014) which can amplify 1500 bp product. Kati et al. (2007) compared nearly complete (1413 nucleotide) 16S rDNA sequence of local Bn1 strain and found 99% similarity with those of previously deposited *B. thuringiensis*

sequences. In the present study, 1500 bp amplicon was obtained using universal bacterial primer which is comparable to the findings of the earlier workers. Sequences obtained following PCR with universal primer set for *Bacillus* were analysed in the present investigation and these were identified as *Bt* by blastn program (Shishir et al. 2012a, Shishir et al. 2012b, Shishir et al. 2014, Tripathi et al. 2016). Sequencing of PCR products further confirmed that these newly isolated *Bacillus* strains that formed parasporal inclusions and exhibited toxicity against lepidopterans were novel isolates of *Bacillus thuringiensis* as supported by similar findings of earlier workers (Assaeedi et al. 2011).

PCR is one among the various tools that has been widely used for amplification and characterization of genes coding for cry proteins and for analysis of *B. thuringiensis* collections (Porcar and Juárez-Pérez 2003). This technique was first introduced by Carozzi et al. (1991) to identify *cry* genes in order to identify insecticidal activity. Over the last decade, PCR including multiplex PCR methods, have been used for screening *cry* genes present in *B. thuringiensis* collections, and to identify strains that harbour genes coding for known *cry* genes (Juárez-Pérez et al. 1997, Bravo et al. 1998, Uribe et al. 2003). It has been reported that insecticidal *cry* genes are normally located in plasmids of larger molecular mass generally more than 30 kb (Gonzales and Carlton 1980, Andrup et al. 2003, Rolle et al. 2005), but it has also been suggested that these genes are present in the main chromosome (Kronstad et al. 1983). Since our isolates could not produce mega plasmid it may be assumed that the toxic gene coding region is associated with the genomic DNA.

Kati et al. (2007) found a fragment with size of approximately 272 bp corresponding to *cry* 1 gene as was reported by Ben-Dov et al. (1997). Whereas Ceron et al. (1995)

and Ozturk et al. (2008) found a fragment of 290 bp for *cry* 1 gene. Bozlağan et al. (2010) found out of 17 *Bt* strains isolated from soil, 6 produced 270bp amplicon using *cry* 1 specific primer . Identification of cry genes by PCR method to know insecticidal properties of the isolates has been successfully done by Carozzi et al. (1991) and Hansen (1998). Ben-Dov et al. (1997) have reported the presence of more than one cry gene in a single isolate with dual activity. Most *Bt* contains complex of *cry* genes (Juárez-Pérez et al. 1997, Bravo et al. 1998, Uribe et al. 2003) but some others can have a single *cry* gene only. The presence of more than one *cry* gene may suggest that *Bt* strain have high frequency of genetic exchange. In present study three virulent strains Arc03, Ab04, Org 6A showed *cry* 1 and *cry* 9 positive amplicons whereas Ab04 showed only *cry* 2 positive amplicon. *Cry* 2 is found to be toxic to lepidopterans and dipterans (Donovan et al. 1988). Apaydin et al. (2005) when screened 163 isolates of *Bt* for *cry* genes, 103 showed positive results for *cry* genes. Among these, 63 isolates had only one type of *cry* gene, 40 isolates had more than one *cry* gene, and 8 isolates had three different *cry* genes.

Patel et al. (2009) analyzed 7 *Bacillus* strains and showed that c*ry* 1 gene was more frequent, while *cry* 9 gene was rather rare. The expected size of *cry* 1 and *cry* 9 genes were ~290 bp and ~350 bp, respectively (Ceron et al. 1995).

Lepidopteran active *cry* 1 proteins are the most studied toxins of *Bt* for their structure and mode of action (Harvey et al. 1986, Choma et al. 1990, Ge et al. 1990, Knowles and Dow 1993, Knowles 1994). The delta endotoxins are encoded by *cry* 1, *cry* 2 and *cry* 9 genes which are toxic to lepidopteran insects (Bravo et al. 1998, Zhong et al. 2000). Specific primers for *cry* 2 genes amplified a 1500 bp amplicon (Sauka et al. 2005) which is evident in the present study too.

Jansens et al. (1997) reported that *cry* 9 was effective in controlling *Plutella xylostella*, which was otherwise resistant to *cry* 1 toxin. Porcar et al. (2000) indicated the importance of other soluble toxins (beta exotoxin) being effective against certain insect.

A high frequency of occurrence of *cry* 1 gene seems to be common in most *B. thuringiensis* strains analyzed so far (Bravo et al. 1998, Hernández et al. 2005). Ben-Dov et al. (1997) detected presence of 49.5% of *cry* 1 gene; Uribe et al. (2003) reported occurrence of 73% *cry* 1 gene; whereas, Wang et al. (2003) detected 76.5% of *cry* 1 gene and 70% of *cry* 2 gene. Thammasittirong and Attathom (2008) reported that strains containing *cry* 1-type genes frequented at 81.3% almost at the same frequency as strains harboring *cry* 2 gene (80.6%). In present study all the three strains showed presence of atleast two *cry* genes (*cry*1 and *cry*9), whereas, Ab04 also contained *cry*2 gene. Therefore, presence of more than one *cry* gene makes the newly isolated strain effective entomopathogens. Therefore, these isolates are good possibilities in the search for biological control agents with a wider spectrum of activity.

7. SUMMARY OF THE Ph.D. THESIS WORK

- Sampling of the host insects (*A. submarginata, A. bipunctata and O. postica*) was done from the tea plantations of Darjeeling foothill region and the adjoining plains (Terai and the Dooars).
- The entomopathogenic bacteria were isolated from the insect cadavers and moribund larvae collected from various tea gardens and tested following a standard procedure and Koch's postulate.
- Three bacterial strains of *Bacillus* Arc01, Arc02, Arc03 from *A. submarginata*; four strains Ab01, Ab02, Ab03 and Ab04 from *A. bipunctata* and two strains Org2A and Org6A from *O. postica* were selected for the study because they were more frequent in the natural population.
- All the morphological characteristics of bacterial isolates such as colony morphology, vegetative body structure, spore-shape presence of parasporal crystals were found to be similar to genus *Bacillus* and were comparable to commercially available *Btk* which was used as a reference in the study.
- Generation/ doubling time and cfu/ml of all the isolates were different thus making newly isolated bacteria different *Bacillus* strains.
- Biochemical tests indicated that all the newly isolated *Bacillus* strains differed with each other and with reference *Btk*, as biochemical test is done with the purpose of comparison of the unknown with the known. Similarity index based on biochemical tests showed highest similarity and dissimilarity among a pair of *Bacillus* isolates.

- In SDS-PAGE of crystal protein of reference strain *Btk*, Arc01, Arc03 and Ab04 showed 128, 128.4, 122.7 and 129.8 kDa bands, respectively, they were the size of ~130 kDa band corresponding to *cry*1/*cry*9 toxin. Additionally, Arc01 had 64 kDa, Ab01 had 56.7 kDa, Ab03 had 56.2 kDa, Ab04 had 56.5 kDa and Org6A had 57.7 kDa band, all correspond to ~60 kDa of *cry*2 toxin gene.
- The whole cell protein analysis of *Bacillus* isolates differed in protein content for different strains, highest for Arc03 (2.356 mg/ml) and lowest for Ab01 (1.094 mg/ml) and corresponding bands ranging from 10 to 126.6 kDa (Arc01, Arc02 and Arc03) 29 to 110 kDa (Ab01, Ab02, Ab03 and Ab04) and 30.8 to 78.5 kDa (Org2A and Org6A). The difference in the whole cell protein content and bands among different *Bacillus* strains and with reference *Btk* indicated that they are distinct strains.
- Apaydin et al. (2005) reported presence of a major plasmid band of 15kb in 33 *Bt* isolates with varying size between 15 kb and 22 kb in *Btk* from Korean soil. In present study too, the size of plasmids ranged from 16 kb (Ab01) to 20.7 kb (Arc01) which is in agreement with the above results. Yilmaz et al. (2013) found 19.3 kb plasmid band comparable to 19.4, 19.8 and 19 kb plasmid bands of Ab04, org 6A and reference *Btk,* respectively, in the present study.
- Low LC_{50} and LT_{50} values of Arc03 , Ab04 and Org 6A indicated their high virulence than *Btk.*
- All the *Bt* strains of bacteria isolated from *A. submarginata, A. bipunctata* and *O. postica* were found to be non- pathogenic to the silk worm larvae. So it can be inferred that the newly isolated bacterial strains, Arc01, Arc02, Arc03, Ab01, Ab02, Ab03, Ab04, Org 2A and Org 6A with proper formulation are

apparently safe for spraying in the tea plantation for controlling the respective pest species.

- The most virulent *Bacillus* species i.e Arc01, Ab04 and Org6A when sequenced for 16S rDNA gene showed 99% similarity with *Bacillus thuringiensis* sequence.
- Sequencing of PCR products further confirmed that these newly isolated *Bacillus* strains that formed parasporal inclusions and exhibited toxicity against lepidopterans were novel isolates of *Bacillus thuringiensis.*

8. CONCLUSION AND DELIVERABLES

- The three main tea growing regions of India are Darjeeling, Assam and Nilgiri. Tea is the main agro-industry of North-East India including the Dooars, Terai and Darjeeling foothill region.
- The tea was first used in China as a medicinal drink and later became a popular stimulating beverage. Nowadays, it has gained popularity world-wide.
- Tea plantation suffer largely from, pest attack. There are approximately, 300 species of insects, mites and nematodes infesting tea, out of which about 25 arthropod species have been recognized as serious pests in North East India.
- To overcome the crop loss, the tea pests are mostly managed conventionally by using synthetic insecticides. Many synthetic insecticides are non-biodegradable, others degrade very slowly and persist in the environment. Insecticides cause pollution of soil and ground water and have harmful effects on a wide range of non-target organisms (beneficial insects, mammals and human).
- Continuous exposures to insecticides, result in development of resistant strains of insect, management of which require increased doses of insecticides or introduction of new insecticides. Different chemical pesticides (organophosphates and synthetic pyrethroids) have been found to be less effective against defoliators in recent time.
- In view of this and also due to a greater acceptance of organic tea (as compared to synthetic chemically managed conventional tea) by health conscious consumers, the future protection and production of tea appear to depend largely on non-conventional control methods.

- One of the ecofriendly approaches of biological control is conservation of the microbial bio-agents or application of some of the effective bacterial control agents of the pest.
- Report of development of insect resistance to commercially available *Btk* has stimulated new research to find additional local *Bt* strains and other microbes that have specific activity spectrum against certain insect pests.
- For knowing the natural occurrence of the entomopathogenic bacteria, population sampling of the host insects (*A. submarginata, A. bipunctata and O. postica*) was done from the tea plantations of Darjeeling foothill region and the adjoining plains (Terai and the Dooars).
- Numerous *Bacillus thuringiensis* subspecies had been isolated from dead and dying insect larvae and in most cases the isolate had toxic activity to the insect from which it had been isolated.
- In this research study of entomopathogenic microbials such as bacteria that naturally infect and kill the pests were surveyed, isolated, and characterized then to determine their efficacy, so that in future, potential microbial pesticides may be developed.
- The entomopathogenic bacteria obtained from larval cadavers of sporadic tea pests, *Arctornis submarginata*, *Andraca bipunctata* and *Orgyia postica* were isolated and tested following a standard procedure and Koch's postulate.
- After characterization, the bacteria were bioassayed on 2^{nd} instar of their respective insect host. The percentage mortality, LC_{50} and LT_{50} values were determined. After bioassay cross-infectivity to the beneficial insect, silkworm was tested.

- Three bacterial strains of *Bacillus* Arc01, Arc02, Arc03 from *A. submarginata*; four strains Ab01, Ab02, Ab03 and Ab04 from *A. bipunctata* and two strains Org2A and Org6A from *O. postica* were selected for study.
- All the newly isolated *Bacillus* strains were found to be different from each other and also from commercial strain of *Bacillus thuringiensis kurstaki* in respect of characteristics and toxicity.
- Molecular characterization of these isolates confirmed that they were indeed strains of *Bacillus.*
- After comparing all the data it may be said that the bacterial strains (*Bacillus* sp. Arc03, Ab04 and Org6A) might be developed in future as potential microbial biopesticides having similar or greater efficacy than *Btk* which is already being used for controlling different lepidopteran tea pests.

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APPENDIX I

INSTRUMENTS	MAKE/ MANUFACTURER
Autoclave	Adhir Dutt and Co., India
Laminar flow hood	Toshiba, India
Micropipettes and tips	Tarson,
Inoculating needle	Tarson
L- Spreader	Tarson,
ELISA plate reader	MRX Revelation, DYNEX
Spectrophotometer	Rayleigh UV-2601
Incubator	Remi,
Phase contrast microscope	Olympus
Camera	MC Camera, Motic, Olympus, Cannon
Cooling centrifuge	Eppendorf
Table top centrifuge	Remi,
Electronic balance	Satorius
PH meter	Hanna
Vortex mixture	Biorad
-20°C freezer	Bluestar,
Refrigerator	Godrej, India
Magnetic stirrer	Remi,
Horizontal electrophoresis unit	Genei, India
Vertical electrophoresis unit	Biotech,
Electrophoresis power supply	Genei, India

UV trans-illuminator	Gibco BRL, Life technologies
Gel documentation system	Spectronics corporation
PCR machine	Eppendorf,
Water bath	Labman scientific instrument
Water distillation plant	Riviera,
Deionised water system	Millipore corporation, USA

APPENDIX II

Composition of different agars

Anaerobic Agar

Ingredients	Amount/1000ml
Trypticase	20g
Glucose	10g
Sodium chloride	5g
Sodium thioglycolate	2g
Sodium formaldehyde sulfoxylate	1g
Agar	15g
Distilled water	1000ml

Adjust the pH to 7.2 distribute into 15 mm test tubes in amounts sufficient to give a 75 mm depth of medium and sterilize by autoclaving at 121°C for 20min.

Nutrient Agar

Ingredients	Amount/1000ml
Nutrient agar	28g
Distilled water	1000 ml

Adjust pH 7.4 and autoclave at 121°C for 15 min.

Nutrient Broth

Ingredients	Amount/1000ml
Nutrient Broth	13g
Distilled water	1000 ml

Adjust pH 7.4 and autoclave at 121°C for 15 min.

Milk Agar

(For Casein hydrolysis)

Ingredients	Amount/1000ml
Casein/skimmed milk powder	100g
Peptone	5g
Agar	15g
Distilled water	1000 ml

Adjust pH 7.2 and autoclave at 121°C for 15 min.

Starch Agar

(For startch hydrolysis)

Ingredients	Amount/1000ml
Peptone	5g
Beef extract	3g
Starch soluble	2g
Agar	15g
Distilled water	1000 ml

Adjust pH 7.0 and autoclave at 121°C for 15 min.

Catalase Agar

(For catalase test)

Ingredients	Amount/1000ml
Tripticase	15g
Phyton/Teptron	5g
NaCl	5g
Agar	15g
Distilled water	1000 ml

Adjust pH 7.0 and autoclave at 121°C for 15 min.

Bacillus thuringiensis maintaining media

Ingredients	Amount/1000ml
Glucose	3g
Ammonium sulphate	2g
Yeast extract	2g
Potassium phosphate dibasic	0.5g
Magnesium sulphate hepta hydrate	0.2g
Calcium chloride dihydrate	0.08g
Manganese sulphate	0.05g
Agar	15g
Distilled water	1000 ml

Adjust pH 7.3 and autoclave at 121°C for 15 min.

Composition of different reagents

Preparation of the various stains used

Crystal violet stain

Solution A: 2g of crystal violet (90%) dye content) was dissolved in 20 ml of ethanol.
Solution B: 0.8g of ammonium oxalate was dissolved in 80ml of distilled water
Solution C: Solutions A and B were mixed together and filtered through tissue paper to remove the particulate matter, if any and stored in a dark bottle at room temperature. Fresh staining solutions were prepared once in two weeks.

Malachite stain

5.8 g of Malachite green was dissolved in 100ml of distilled water and mixed thoroughly. Strained and filtered and stored at room temperature.

Safranin stain

2.5 g of Safranin was dissolved in 100 ml of 95 per cent ethanol and from this 5% aqueous solution was prepared and stored at room temperature.

Gram's stain

Gram's iodine: Dissolve 0.33 g of iodine and 0.66 g of potassium iodide in 100 mL of distilled water; alternately, dilute 0.1 (N) iodine in 1:4.

Counter stain stock solution: Dissolve 2.5 g of certified safranin in 100 mL of 95% ethyl alcohol.

Composition of different buffers and dye for Electrophoresis

Agarose gel electrophoresis

TAE buffer (50 X)

Tris base	242 g
Glacial acetic acid	57.1 ml
EDTA 0.5 M (pH 8.0)	100 ml

Tris-Glycine running buffer (10X)

Tris base	30.0g
glycine	144.0g
SDS	10.0g
Distilled water	1000ml

The pH of the buffer should be 8.3.

Loading dye (5 X)

Bomophenol blue	0.25%
Xylene cyanol	0.25%
Glycerol	30%

Sample Loading Buffer (5X)

Tris·HCl (pH 6.8)	250 mM
SDS	10%
Glycerol	30% (v/v)
DTT	10 mM
Bromophenol Blue	0.05% (w/v)

APPENDIX III

LIST OF PUBLICATIONS

Ananda Mukhopadhyay, Soma Das, Ritesh Biswa, **Sangita Khewa (Subba),** Anjali Km Prasad, Kumar Basnet, Jayashree Saren and Mrinal Ray. 2015. Tea pests of Terai-Dooars, Implication of their conventional control and sustainable management options-an overview. ***Global Journal of Environmental Science and Research***. 2(3): 93–106.

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APPENDIX IV

LIST OF ABSTRACT PUBLISHED IN PROCEEDINGS OF CONFERENCES/ SEMINAR/ WORKSHOPS

Sangita Khewa (Subba), Pali Dhar and Ananda Mukhopadhyay. 2008. A glimpse of the campus birds of North Bengal University. Silver Jubilee Symposium on **Dimension of Research Application in Animal Sciences**, Dept of Zoology University of North Bengal. **(Oral presentation)**

Sangita Khewa (Subba) and Ananda Mukhopadhyay. 2009. Biocontrol potential of a newly isolated bacterial agent against *Arctornis submarginata* (Walker) (Lepidoptera:Lymentriidae) occurring in Darjeeling Terai region. **2nd International BIOCICON 2009**, Crop Protection Research Centre (CPRC), Dept of Advance Zoology and Biotechnology, St. Xavier's College (Autonomous), Palayamkottai, Chennai. **(Poster presentation)**

Sangita Khewa (Subba) and Ananda Mukhopadhyay. 2010. Comparison of entomopathogenic bacterial strains isolated from white grub, *Phyllognathus dionisius* (Fabricius) (Coleoptera: Dynastiidae) occurring in tea plantations of Darjeeling foothills. **Evaluation of Biodiversity of Eastern Himalaya and Adjoining Plains**, Department of Zoology, University of North Bengal. **(Oral presentation)**

Sangita Khewa (Subba) and Ananda Mukhopadhyay. 2011. Characterizing and testing biocontrol potential of two *Bacilllus* strains isolated from the tea defoliating pest, Arctornis submarginata (Walker) (Lepidoptera: Lymantriidae). **Biodiversity & Food security- Challenges & Devising strategies** organized by Dheerpura society for advancement of science and rural development and Indian institution of pulses research, Kanpur (UP), India. **(Poster presentation)**

Sangita Khewa (Subba) and Ananda Mukhopadhyay. 2012. Isolation and characterization of two *Bacillus* strains toxic to *Hyposidra talaca* (Walker) (Lepidoptera:

Geometridae) from Darjeeling foothill region**. PLACROSYM XX**, UPASI Tea Research Foundation, Tea Research Institute, Valparai, Coimbatore, Tamil Nadu. **(Poster presentation)**

Sangita Khewa (Subba) and Ananda Mukhopadhyay. 2014. Isolation of Bacillus strains from Bunch caterpillars of Darjeeling Tea Andraca bipunctata (Walker) (Lepidoptera: Bombycidae) with bioassay of their host pathogenicity. AZRA Silver Jubilee International Conference on **Probing biosciences for food security and environmental safety**, Central Rice Research Institution, Cuttack, Odisha. **(Oral presentation)**

Sangita Khewa (Subba) and Ananda Mukhopadhyay. 2014. Characterization and evaluation of entomopathogenicity of two *Bacillus* strains isolated from the sporadic tea pest, *Orgyia postica* (Walker) (Lepidoptera: Lymantriidae) of Darjeeling hills. National Symposium on **Plant Protection in Tea: Recent Advances**, Tea Research Association, Toklai Tea Research Institute, Assam. **(Poster presentation)**

Advances in Life Sciences 1 (2): 114-117, 2012

Characterizing and Testing Biocontrol Potential of Two *Bacillus* Strains Isolated from the Tea Defoliating Pest, *Arctornis submarginata*(Walker) (Lepidoptera: Lymantriidae)

SANGITA KHEWA (SUBBA)[1] AND ANANDA MUKHOPADHYAY[2]

Entomology Research Unit, Department of Zoology, University of North Bengal, Darjeeling, 734 013
e-mail: sangita.khewa@yahoo.com[1], am_nbu@yahoo.co.in[2]

ABSTRACT

Two strains of *Bacillus* were isolated from the diseased caterpillars of *Arctornis submarginata* (Walker) (Lepidoptera: Lymantriidae), a defoliator of tea crop from Darjeeling Terai region. Biochemical characteristics of *Bacillus* strain Arc02 showed positive reaction in lysine utilization, ornithin utilization, Citrate utilization, malonate utilization, esculin hydrolysis, rhamnose, cellobiose, raffinose and glucose utilization while Arc03 strain showed positive reaction in nitrate reduction, H_2S production, V-P test, esculin hydrolysis and utilization of citrate, saccharose, trehalose and glucose. Studies on SDS-PAGE of crystal protein of of Arc 02 showed four major protein bands with the molecular weights 117.32 kDa, 75.14 kDa ,55.97 kDa and 39.15 kDa, while the profile of four protein bands of Arc03 showed 122.7 kDa, 85.8 kDa, 50.3 kDa and 46.84 kDa bands. The LC_{50} value was of Arc02 was found to be 796.22µg/ml compared to 342µg/ml as the LC_{50} value of Arc03. Based on LC_{50} value the latter strain (Arc03) apprears to be more toxic with a higher biocontrol potential as a microbial pesticide against *A.submarginata*.

Key words: *Bacillus strain Arc02 and Arc03, Arctornis submarginata, Defoliating pest, Tea, Darjeeling.*

Tea is the main agro-industry of Darjeeling hills and its adjoining plains of the Doars, Terai and the North-East region of India. But the plantation of this region is often damaged by attacks of lepidopteran tea pests. Among which *Arctornis submarginata* commonly called as hairy caterpillar is emerging as a potential pest (Mukhopadhyay and Roy, 2009). It defoliates the mature maintenance leaves hampering both quality and quantity of tea (Mukhopadhyay *et al*., 2007). To combat the present day pest problem chemical pesticides are mainly used with some back lashes such as environmental pollution, human health hazard, resistance in pests (Sarker and Mukhopadhyay, 2006). In view of this, efforts are being made to evolve alternative strategies of management of these pests through application of microbial bioagents. Development of microbial pesticides would greatly help in production of export quality tea through biorational or organic farming. In an earlier study occurrence of a strain of *Bacillus* (Arc01) was recorded from a population of *A. submarginata* (Khewa and Mukhopadhyay, 2010). To explore more entomopathogenic bacterial strains from the pest caterpillars the present study was undertaken. Isolation and characterization of the naturally occurring bacterial pathogen of *A. submarginata* were done with an objective to find their biocontrol potential through determination of median lethal concentration and time.

MATERIALS AND METHODS

Bacterial strains were isolated after the method of Lacey and Brooks 1997 and stored at -20°C for further analysis. After centrifuging at 3000 rpm for 30 min the precipitate mainly containing bacteria was taken for pure culture isolation by 'dilution streak method' in nutrient agar medium. The infectivity of the isolated bacterial strains were determined following Koch's postulates by infecting healthy first instar larvae.

Cell, spore shape and structure of crystal protein were observed in the isolated bacteria under phase contrast microscope (100X) (Olympus, CX31) and the same were compared with *Bacillus thuringiensis kurstaki* (*Btk*).The colony texture and motility of the bacteria were also determined.

Biochemical analyses like indole, Voges-proskour, methyl red, citrate utilization, esculin hydrolysis, lysine decarboxylase, ornithin decarboxylase, H_2S production, nitrate reduction, fermentation of different carbohydrates, urease tests were performed using Biochemical testing kit (KB003) (Himedia) with *Btk* as reference.

Growth of the isolated bacterial strains were determined by turbidimetric method (Cappuccino and Sherman, 1996). The OD value was taken at 540 nm in spectrophotometer at 30 minutes interval. Doubling time of the strains were determined and compared with *Btk*.

For SDS-PAGE profile of crystal protein 4ml sporulated culture of each isolate was pelleted down at 8000 rpm for 3 minutes and re-suspended in 50µl sterile distilled water. 5 µl of 1N NaOH was added after vortexing and the mixture was incubated for 5 minutes. 15 µl of Laemmli's (2x) buffer was added to it and boiled for 2 mins. SDS PAGE was run after the method of Lammeli 1970 and followed by Moraga *et al*., 2004. The bands of crystal proteins were analysed using gel documentation system (Spectroline, model no. TVD-1000R/F).

Likewise SDS-PAGE analysis of whole-cell proteins was done after Costas 1992. The bacteria were cultured on Luria-Bertani (LB) agar for 24h at 37°C and the proteins were extracted using 1% lysozyme solution and lysis buffer containing 4% SDS, 20% glycerol, 2% 2-marcaptoethanol, 70% Tris-HCL, P^H 6.8, and 4% deionized water. Gel was run after the method of Lammeli 1970 and followed by Morga. The bands of whole-cell proteins were analysed using gel documentation system (Spectroline, model no. TVD-1000R/F).

Crude spore crystal mixture (100, 300, 500, 750 and 1000 ig/ml) of the bacteria (isolated from *A.submarginata*) and that of *Btk* were used for bioassay after the method of Unnamalai and Sekar 1995. Tea leaves dipped in different concentrations of the said mixtures were offered as food to the second instar larvae (n=100) of *A. submarginata*. Leaves dipped in sterile distilled water were used in control. The mortality was observed at 24h interval after their exposure. Median lethal concentration (LC_{50}) value was determined by Probit analysis after Finney (1954). Value of median lethal time (LT_{50}) was also determined following the method of Biever and Hostetter (1971).

RESULTS AND DISCUSSION

Bacterial strains Arc02 and Arc03 showed similarity in all the morphological characteristics. Their vegetative body structure (chain like), spore-shape (oval), presence of cryatals, motility (high), colony texture (smooth), were found to be similar among themselves and with that of *Bacillus thuringiensis kurstski* (*Btk*) (Fig1 and 2). The isolated strains showed characteristics of genus *Bacillus* such as rod shaped vegetative body, endospore formation, Gram positivity, facultative anaerobic nature, catalase positivity, acid production from glucose and motility (Sneath, 1986). Biochemical characteristics of Arc02 strain showed positive reaction in utilization of lysine, ornithin, Citrate, malonate, , rhamnose, cellobiose, raffinose, glucose and esculin hydrolysis. It showed difference with *Btk* in ONPG test, V-P test, nitrate reduction, esculin hydrolysis and in utilization of

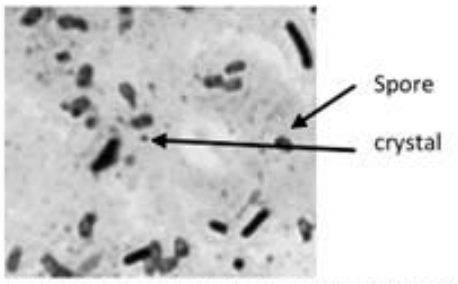

Figure 1. Spores and crystals of *Bacillus* strain Arc02 (Magnification 100X).

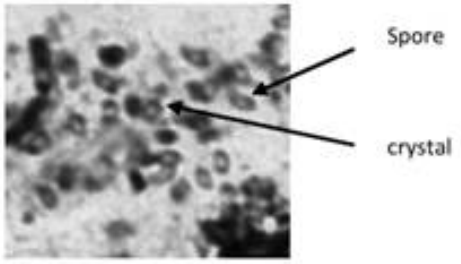

Figure 2. Spores and crystals of *Bacillus* strain Arc03 (Magnification 100X).

citrate, malonate, arabinose, xylose, rhamnose, melibiose, saccharose, raffinose and trehalose. Likewise, Arc03 strain showed positive reaction in nitrate reduction, H_2S production, V-P test, esculin hydrolysis and utilization of citrate, saccharose, trehalose and glucose. It showed difference with *Btk* in ONPG test, H_2S production, esculin hydrolysis and

Table 1. Biochemical characteristics showing difference in *Bacillus* strains Arc 02, Arc 03 and *Btk*

Biochemical Characteristics	*Bt kurstaki (Btk)*	Arc 02	Arc 03
ONPG	+	-	-
Lysine carboxylase	+	+	-
Ornithin decarboxylase	+	+	-
Urease	-	-	-
Phenyl alanine deamination	-	-	-
TDA			
Nitrate reduction	+	-	+
H_2S production	-	-	+
Citrate utilization	-	+	+
V-P Test	+	-	+
Methyle red	-	-	-
Indole	-	-	-
Malonata	-	+	+
Esculin hydrolysis	-	+	+
Arabinose	+	-	-
Xylose	+	-	-
Adonitole	-	-	-
Rhamnose	-	+	-
Cellobiose	+	+	-
Melibiose	+	-	-
Saccharose	+	-	+
Raffinose	-	+	-
Trehalose	+	-	+
Glucose	+	+	+
Lactose	-	-	-
Oxidase	-	-	-

utilization of lysine, ornithin, citrate, malonate, arabinose, xylose, rhamnose and melibios. (Table 1).

The doubling time was 20 min in case of Arc 02, 35 min in case of Arc 03 which were lower than that of *Btk* which had doubling time of 42 min.

When composition of crystal protein was analyzed by SDS-PAGE, crystals of of Arc 02 showed four major protein band having the molecular weight 117.32 kDa, 75.14 kDa, 55.97 kDa and 39.15 kDa which in case of *Btk* were 115.82 kDa, 44.4 kDa and 29.55 kDa protein bands. So, a sharp difference in banding pattern was found between Arc 02 and *Btk* . In Arc 03 again four protein bands having molecular weight 122.7 kDa, 85.8 kDa, 50.3 kDa and 46.84 kDa were found which was different from the bands found in Arc 02 and *Btk*. (Fig 2). SDS-PAGE of the whole-cell protein of Arc 02 showed three major protein band having the molecular weight 126.69 kDa, 43 kDa, and 27.72 kDa which in case of *Btk* were 110.5 kDa, 55.34 kDa, 40.51 kDa and 25.82 kDa. Arc03 had four major bands having molecular weight 124.06 kDa, 85.8 kDa, 50.31 kDa and 42.99 kDa. The above two strains showed difference in their banding pattern which also differed from that of *Btk*. (Fig. 3)

When the dose (100 to 1000 µg/ml) dependent mortality of the second instar *A. submarginata* larvae was recorded, it was observed that in Arc02 the percent mortality varied from 12% to 63% within 9 days. The LC_{50} value was found to be 796.22µg/ml with fiducial lower limits 662.5µg/ml and upper limit 957µg/ml. The LT_{50} value was 5.5 days for 1000 µg/ml concentration but no 50% mortality was observed in lower concentration. In case of Arc03 strain of *Bacillus* the percent mortality for different doses (100 to 1000 µg/ml) of second instar *A. submarginata* larvae varied from 26% to 79% within 9 days. The LC_{50} value was calculated as 342µg/ml with fiducial

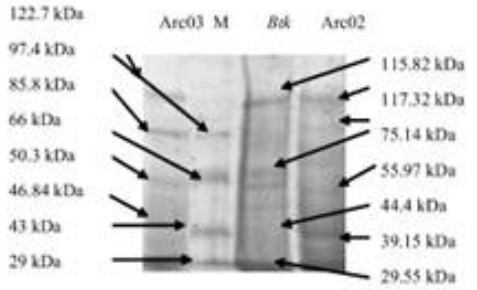

Figure 3. SDS-PAGE of crystal protein of *Bacillus* strain Arc02, Arc03 and *Btk*.

lower limits 281µg/ml and upper limit 414.99µg/ml. The LT_{50} values were 5.28 days for 1000 µg/ml, 6.3 days for 750 µg/ml 7.42 days for 500 µg/ml concentrations. *Btk* showed LC_{50} value as 537 µg/ml with fiducial lower limit 489.63µg/ml and upper limit 590.37µg/ml. The LT_{50} values were 7.57 days for 1000 µg/ml and 9.5 days for 750 µg/ml (Table 2).

The two entomopathogenic bacteria isolated from *A. submarginata* showed difference with *Btk* (commercially used bacterial pesticide) in biochemical tests, generation time and median lethal concentration and time. Therefore may be considered as entomopathogenic strains of *Bacillus* reported for the first time (Khewa and Mukhopadhyay, 2010) and with the designation as 'Arc 02' and Arc 03. These two new strains also differed in most of the parameters from the earlier reported strain Arc01.

The low LC_{50} and LT_{50} values of 'Arc 03' makes it a potential entomopathogenic bacterium to be developed as

Table 2. Bioassay of *Bacillus* strain Arc 02, Arc 03 and *Btk* on second instar *A. submarginata* caterpillars

Bacillus Strain	Concentration (µg/ml)	% mortality	LC_{50}	Lower Fiducial limit	Upper Fiducial limit	Regression	LT_{50}
Btk	1000	76	537 µg/ml	483.63 µg/ml	590.37 µg/ml	Y=0.839 + 1.520x	7.57 days for 1000 µg/ml
	750	60					9.5 days for 500 µg/ml
	500	48					
	300	19					
	100	17					
New isolate of *Bacillus* Arc 02	1000	63	796.22 µg/ml	662.5 µg/ml	957 µg/ml	Y=1.477 + 2.901x	5.5days for 1000 µg/ml
	750	47					
	500	31					
	300	24					
	100	12					
New isolate of *Bacillus* Arc 03	1000	79	342.00 µg/ml	281.70 µg/ml	414.99 µg/ml	Y=2.533 + 1.365x	5.28days for 1000 µg/ml
	750	67					6.3 days for 750 µg/ml
	500	53					7.42 days for 500 µg/ml
	300	44					
	100	26					

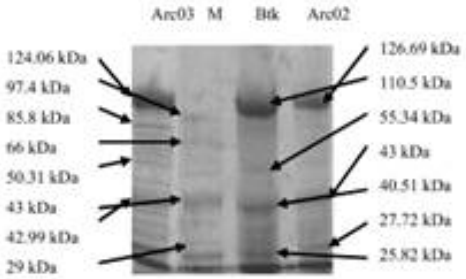

Figure 4. SDS-PAGE of whole-cell protein of *Bacillus* strain Arc02, Arc03 and *Btk*.

microbial pesticide for controlling the caterpillars of *A. submarginata*. Although the LC_{50} value for Arc02 was recorded higher compared to *Btk* but its much lower LT_{50} than *Btk* makes it also promising as biopesticide.

So, the discovery of these new bacterial strains that has been found effective in inducing fair mortality in the defoliator of tea needs to be further developed into biopesticide through better formulation, toxicity assessment and biosafety testing. Future development, improvement and applications of these potential biopesticides that comprise a part of the rich microbial diversity of North East India and in particular that of the tea plantations of Darjeeling foothills and plains will ensure an effective control of tea pest in organic plantations without the use of chemical pesticides.

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Received on 12.05.2012 *Accepted on 15.07.2012*

Global Journal of Environmental Science and Research
ISSN : 2349-7335
Volume 2, Number 3 (Oct.-Dec. 2015)

TEA PESTS OF TERAI-DOOARS, IMPLICATION OF THEIR CONVENTIONAL CONTROLAND SUSTAINABLE MANAGEMENT OPTIONS - AN OVERVIEW

Ananda Mukhopadhyay*[1], Soma Das[1], Ritesh Biswa[1,2], Sangita Khewa Subba[1,3], Anjali Km Prasad[1], Kumar Basnet[1], Jayashree Saren[1], Mrinal Ray[1]

[1] *Entomology Research Unit, Department of Zoology, University of North Bengal, P.O. NBU, Siliguri, Darjeeling, West Bengal-734 013, Email: entoananda@gmail.com*

[2]*Department of Zoology, Gorubathan Govt. College, P.O. - Fagu, Gorubathan, Darjeeling - 735 231*

[3]*Department of Zoology, Siliguri College, Siliguri, Darjeeling - 734 001*

ABSTRACT

Tea being a perennial monoculture plantation crop provides a range of habitat for a number of arthropod pests such as looper caterpillars, tea mosquito bugs, red spider mites, termites and various other sporadic ones. A substantial crop loss is reported regularly by the infestation of these pests in the tea plantations of Terai-Dooars of North East (NE) India. Conventionally, synthetic chemicals belonging to groups like organochlorines, organophosphates, synthetic pyrethroids, carbamates, etc. are used to manage these pest populations. A frequent and desultory application of synthetic pesticides has often resulted in development of higher tolerance, even resistance amongst the certain pest species. Therefore, the usual recommended doses of pesticide are found to be ineffective in managing some of these pests, warranting either increased doses of pesticide use or new group of pesticides. The after effects of such application on non-target organisms and environment are not yet properly assessed. The other major concern is the increased MRL in the made tea which is hampering the export potential of Indian tea. Therefore, various non-conventional pest management strategies are being attempted and developed in the recent years such as use of microbial and botanical formulations, conservation of parasitoids and predators and their *in vivo* production on artificial diet-reared hosts (pests), besides other manual/ cultural control practices such as hand collection of pests, light trapping, sticky trapping, management of alternative hosts etc. Expectedly these practices can be effective in reducing the pesticide load in tea plantations of NE and also help in planning an environment friendly IPM strategy.

Key words : Tea-associated arthropods, pesticide tolerance, non-conventional control, artificial diet rearing

Tea pest diversity and management

Tea, the foliage crop, cultivated as long term monoculture provides an ambience for greater colonization by arthropods with more and more growth and branching of the bush frame and periodic setting in of new leaf. The advantage of tea as a model plant is that being perennial it allows a quick build-up of phytophages of diverse feeding habits (Banerjee 2009) and as such appears ideal for a study of diversity and spatial dynamics of phytophagous arthropod guilds and their associated natural enemies. Starting from root, stem, both maintenance and pluckable leaves, flowers and seeds are infested by various pest species such as loopers, tea mosquito bugs, red spider mites, red slug, hairy caterpillars, termites, grubs, stem-hole borers etc. resulting in 11%-55% loss in yield if left unchecked (Hazarika et al. 2009). Tea plantations of North East (NE) India harbour around 173 arthropods and 16 nematodes as the pest species (Hazarika et al. 1994) among them, the majority (167) are insects (Mukhopadhyay and Roy 2009).

An analysis of the tea arthropod variety and variability of Terai-Dooars regions of Himalayan foothills indicates succession cycles of guilds. It starts

*Corresponding Author

Research Article

NBU J. Anim. Sc. 8: 23–31 (2014)
ISSN 0975-1424

CHARACTERIZATION AND EVALUATION OF ENTOMOPATHOGENICITY OF TWO *BACILLUS* STRAINS ISOLATED FROM THE SPORADIC TEA PEST, *ORGYIA POSTICA* (WALKER) (LEPIDOPTERA: LYMANTRIDAE) OF DARJEELING HILLS

Sangita Khewa Subba* and Ananda Mukhopadhyay
Entomology Research Unit, Department of Zoology, University of North Bengal, Darjeeling-734013

Received: November 13, 2014 **Revised & Accepted**: December 28, 2014

ABSTRACT

Two strains of entomopathogenic bacteria were isolated from the diseased caterpillars of *Orgyia postica* (Walker) (Lepidoptera: Lymantridae) collected from the tea plantations of Darjeeling hill slope. Two bacterial isolates Org2A and Org6A, on the basis of the biochemical and physiological tests *viz.* cell morphology, gram positivity, endospore production, facultative anaerobic nature, hydrolysis of starch, catalase positivity, production of acid from glucose and motility etc, the two strains could be assigned to the genus *Bacillus, viz.* cell morphology, gram positivity, endospore production, facultative anaerobic nature, hydrolysis of starch, catalase positivity, production of acid from glucose and motility etc. *Bacillus thuringiensis kurstaki.* Further difference between the isolated *Bacillus* strains (Org 2A and Org 6A) and standard strain (*Btk*) was observed in molecular weight of their crystal protein on SDS-polyacrylamide electrophoregram. Crystals of both Org2A and Org 6A showed one major protein band having the molecular weight 36.5 kDa and 60.7 kDa respectively, while contrastingly *Btk* showed three bands of 110.7, 56.6 and 30.2 kDa. Further, in bioassay testing against *O. postics*, the LC_{50} value of Org 2A and Org 6A against *O. postica* larvae was found to be 543.39µg/ml and 354.87µg/ml, respectively. And for *Btk*, the LC_{50} value was found to be 386.21µg/ml. Thus, Org 6A appears to be more virulent than the commercially used biopesticide, *Btk*. The result indicates availability of two novel strains of *Bacillus* having potential to control the tea attacking caterpillars of *O. postica*.

Keywords: *Bacillus* strains Org 2A, Org 6A, *Orgyia postica*, Tea, Darjeeling.

INTRODUCTION

The tea industry is one of the oldest organized industries in India and Indian teas are appreciated world over as refreshing beverage for their unique flavor, aroma and medicinal properties. India contributes about 27.49 % of global tea production of which 23% is produced by North Bengal (Darjeeling hills and its adjoining plains (the Dooars and Terai) (Anonymous, 2003). The beverage- crop tea, [*Camellia sinensis* (L.) O. Kuntze] is the economic backbone of North Bengal, but tea plantations of this region is severely damaged by attacks of lepidopteran pests. One of the sporadic lepidopteran defoliators is the caterpillars of *Orgyia postica* (Walker). The pest was reported from North-East India as early as 1930s by Watt and Mann. This pest species is also known to cause considerable damage to the tea bushes of Darjeeling foothills and terai region (Pathak *et al.*, 2003). Populations of defoliating pests have so far been controlled by regular application of synthetic pesticides especially organophosphates and pyrethroids, but with time the pests have become less susceptible, often resulting in control failures (Sannigrahi and Talukdar, 2003; Sarkar and Mukhopadhyay, 2006). Moreover, health conscious consumers prefer organic tea to the one produced conventionally with application pesticides. Though

***Author for correspondence:** Sangita Khewa Subba, Email: sangita.khewa@yahoo.com

J. Appl. Zool. Res. (2014) **25**(1):39-44

ISOLATION AND CHARACTERIZATION OF TWO *BACILLUS* STRAINS TOXIC TO *HYPOSIDRA TALACA* (WALKER) (LEPIDOPTERA: GEOMETRIDAE) FROM DARJEELING FOOTHILL REGION

SANGITA KHEWA (SUBBA)[1], ANANDA MUKHOPADHYAY[1] AND DAMAYANTI DE[2]

Received: 30 January, 2014 **Accepted: 30 April, 2014**

ABSTRACT: Two strains of *Bacillus* were isolated from the diseased caterpillars of *Hyposidra talaca* (Walker) (Lepidoptera: Geometridae), a defoliator of tea crop from Darjeeling Terai region. Biochemical characteristics of *Bacillus* strain HT01 and HT02 showed positive difference with *Btk*. SDS PAGE of the crystal protein of HT01 showed the presence of three distinct protein bands having molecular weight 93.3 kDa, 73.8 kDa and 59.1 kDa. HT02 showed three distinct bands of molecular weight 95.4 kDa, 59.4 kDa and 41.2 kDa. Banding pattern of crystal protein of *Bacillus thuringiensis kurstaki* (*Btk*) differed in the banding pattern with both HT01 and HT02 in having three distinct bands of 96.6kDa, 74.9kDa and 60.7kDa. Further, the plasmid lengths of HT01 and HT02 also differed significantly with each other having the value of 19.08 kbp and 21.56 kbp respectively. The LC_{50} value for HT01 strain was calculated as 199.83 µg/ml compared to the LC_{50} value for HT02 strain which was found to be 271.56µg/ml. Both HT01 and HT02 showed lower LC_{50} value than *Btk* i.e. 438.19µg/ml when tested on *H. talaca* larvae. Based on LC_{50} value the new strains (HT01 and HT02) appear to be more toxic with a higher biocontrol potential as a microbial pesticide against *H. talaca*.

Key words: *Bacillus* strain HT01 and HT02, *Hyposidra talaca*, Defoliating pest, Tea, Darjeeling.

INTRODUCTION

The main agro-industry of Darjeeling hills and its adjoining plains (Duars and the Terai) is Tea, *Camellia sinensis* (L.) O. Kuntze, but the plantation of this region is severely damaged by the attacks of lepidopteran pests. Among the lepidopterans attacking tea, *Buzura suppressaria* Guen (Lepidoptera: Geometridae) was reported as a major tea looper about a century back (DAS, 1965). Although its presence has been recorded in tea plantations of Darjeeling and Terai and the Dooars in the last six decades (ANONYMOUS, 1994; SARKER and MUKHOPADHYAY, 2006), recent studies revealed that another polyphagous looper species *H. talaca*, (Walker) (Lepidoptera: Geometridae), commonly known as black inch worm has taken over as the major pest. This looper species feeds on a number of forest plants and weeds from India, Malaysia and Thiland (BROWNE, 1968; MATHEW *ET AL.*, 2005; WINOTAI *et al.*, 2005; DAS and MUKHOPADHYAY, 2008; www.mothsofborneo.com) and has turned to tea, as the most active defoliating insect in tea plantations of Darjeeling Terai and the Dooars (BASU MAJUMDAR and GHOSH, 2004; DAS *et al.*, 2010).

Populations of looper pests of tea have so far been controlled by regular application of synthetic insecticides especially organophosphates and pyrethroids, but with time the pests have become less susceptible, often resulting in control failures (SANNIGRAHI and TALUKDAR, 2003). Since the use of synthetic pesticides is being discouraged in tea due to residue problems and safety concerns of human health and the

[1] Entomology Research Unit, Department of Zoology, University of North Bengal, Darjeeling, 734 013, India

[2] Department of Molecular Cellular and Developmental Biology 306, Kline Biology Tower, Yale University, 266, Whitney Avenue New haven, CT-06520, USA[2] *E-mail: am_nbu@yahoo.co.in*[1]: *damayantitamanna@gmail.com*[2]

maturity of the crop. The infestation of the pest was recorded by Lal and Sachan (1987) on greengram.

Correlation co-efficient values (Table 1) indicated that the larval population of pink pod borer exhibited a significant negative correlation with evening relative humidity (r=-0.561). Whereas, other factors did not show any significant impact on incidence of the pest. Sahoo and Patnaik (1994) reported that maximum temperature and morning relative humidity were found most favourable for buildup of pink pod borer population during pod formation to grain development stage.

The result obtained during the present study indicated that the pink borer started its activity from pod formation stage and remained continued up to maturity crop.

Table 1 : Correlation between weather and pod borers

Population	Weather parameters									
	Temperature (°C)			Relative humidity (%)						
	Max.	Min.	Mean	Morning	Evening	Mean	Wind Speed (km/hr)	Mean bright hours	Rain fall	Rainy days (mm)	Evaporation
Pink Pod Borer	0.208	-0.406	0.047	-0.328	-0.561*	-0.518	-0.417	0.364	-0.326	-0.440	0.263

*Significant at 5% level. (r = ± 0.553) n=13.

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Occurrence of Baculovirus in Black Inch Worm, *Hyposidra talaca* (Walker) (Lepidoptera : Geometriidae) from Darjeeling

Ananda Mukhopadhayay, Damayanti De and Sangita Khewa (Subba)
Entomology Research Unit, Department of Zoology, University of North Bengal, Darjeeling (W.B.,), India

In recent years, besides *Buzura suppressaria* Guenee one more looper species, specially the black inch worm, *Hyposidra talaca* (Walker) have been found to cause extensive damage by defoliating the tea plantation of Terai and the Dooars at the Darjeeling foothills (Basumajumdar and Ghosh, 2004; Das and Mukhopadhyay, 2008). As use of synthetic pesticides is being discouraged on tea due to residue problem and safety of health and environment, naturally occurring populations of *H. talaca* showing pathogenic symptom of viral infection were screened for isolating the causative agent for its future development as biopesticide.

Infected larvae of *H. talaca* turned pale and flaccid with putrification and liquefaction of tissue, and usually hung head down from tea twigs (Fig. 1). Screening of such larvae was conducted for isolating baculovirus after the

International Journal of Tropical Insect Science Vol. 31, No. 1–2, pp. 13–19, 2011
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doi:10.1017/S1742758411000026

Characteristics and virulence of nucleopolyhedrovirus isolated from *Hyposidra talaca* (Lepidoptera: Geometridae), a pest of tea in Darjeeling Terai, India

Ananda Mukhopadhyay*, Sangita Khewa (Subba) and Damayanti De

Entomology Research Unit, Department of Zoology, University of North Bengal, Darjeeling 734 013, India

(Accepted 14 January 2011; First published online 1 June 2011)

Abstract. Nucleopolyhedrovirus (NPV) was isolated from infected *Hyposidra talaca* (Walker) caterpillars. This defoliating pest was sampled from the Terai and Dooars tea plantations along the foothills of Darjeeling, India. Phase contrast and transmission electron microscopy revealed polyhedral occlusion bodies (OBs) typical of NPVs. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis analysis showed that the molecular weight of the major protein of the OBs is 32.17 kDa. Dose–mortality bioassays conducted for OBs on the second instar caterpillars of *H. talaca* resulted in a median lethal concentration (LC_{50}) of 2.8×10^3 OBs/ml. The median lethal time (LT_{50}) was 5.45 days for 1×10^4 OBs/ml, 4.15 days for 1×10^5 OBs/ml and 4.05 days for 1×10^6 OBs/ml concentrations. These results indicate the potential of using this NPV as a microbial pesticide against *H. talaca*.

Key words: *Hyposidra talaca*, nucleopolyhedrovirus, *Camellia sinensis*, Darjeeling

Introduction

The black inch looper *Hyposidra talaca* (Walker) (Lepidoptera: Geometridae) attacks and defoliates mature tea *Camellia sinensis* (L.) O. Kuntze in the foothills of Darjeeling and Terai and the Dooars of northeastern India (26° to 27°13′ North latitude and 87°59′ to 88° 53′ East longitude). In recent years, the looper activities have largely increased. Although *Buzura suppressaria* Guen. (Lepidoptera: Geometridae) has been recorded in tea plantations of Darjeeling and Terai and the Dooars in the last six decades (Anonymous, 1994), recent studies have revealed the incidence of other looper species such as *H. talaca* as a common defoliating insect in tea plantations of Darjeeling Terai and the Dooars (Basu Majumdar and Ghosh, 2004; Das *et al.*, 2010*a*). *H. talaca* usually undergoes four or more overlapping generations throughout the year. Maximum damage occurs during the pre-monsoon period (March–June) followed by a reduction in population during the peak monsoon months (Das and Mukhopadhyay, 2009). A residual population was also recorded during the winter months (Das *et al.*, 2010*b*).

Populations of looper pests of tea have so far been controlled by regular application of synthetic insecticides especially organophosphates and pyrethroids, but with time the pests have become less susceptible, often resulting in control failures (Sannigrahi and Talukdar, 2003; Sarker and Mukhopadhyay, 2006). Since the use of synthetic pesticides is being discouraged in tea due to residue

*E-mail: am_nbu@yahoo.co.in

Exploring the biocontrol potential of naturally occurring bacterial and viral entomopathogens of defoliating lepidopteran pests of tea plantations

Ananda Mukhopadhyay*, Damayanti De and Sangita Khewa

ABSTRACT

The foliar crop tea (*Camellia sinensis* O'Kuntz), that yields the cheapest beverage in India is largely produced in North-East India including the Darjeeling Himalayan slope and its Terai (foothills and plains). Naturally occurring entomopathogenic bacteria could be isolated from two species of loopers, *Buzura suppressaria* and *Hyposidra talaca* and also from a slow but steady leaf feeder, the red slug caterpillar (*Eterusia magnifica*) and a new invading hairy caterpillar (*Arctornis submarginata*) that strip tea bushes of their mature and maintenance leaves. Infected larvae of *B. suppressaria* and *H. talaca* yielded spore forming bacteria with crystal proteins and appeared to share many features in common with *Bacillus thuringiensis kurstaki* (*Btk*), but differed from *Btk* in shape of their crystal, biochemical tests, growth phase, molecular weight of crystal protein, and major whole body proteins. A lower LC_{50} and reduced LT_{50} value than *Btk* were also evident. All the *Bacillus* str ains i. e. BS01 from *B. suppressaria*, HT01 and HT02 from *H. talaca* were found mutually cross infective to both the looper species but were not pathogenic to *Bombyx mori* (multi voltine strain of silkworm). Field application in RBD of most pathogenic strain of HT01 proved alone to be quite effective at the field dose of 5000 µg/ml concentration. Mean live larvae of *H. talaca* recovered in field after 7 days of spraying the bacterial formulation, was 3.93% . So, the present study revealed that naturally occurring highly pathogenic *Bacillus* strains could be made effective in looper control through a process of isolation, identification, testing, formulation and application especially in biorational or bio-organic tea plantations. Bacterial strain close to *Btk* but with a slightly higher LC_{50} value and a much reduced LT_{50} values could be isolated from red slug caterpillars of *E. magnifica*. Its laboratory based evaluation proved its killing efficacy in the early stage red slug caterpillars. So, this strain of *Bacillus* also hold a substantive promise ofbeing used in biocontrol of the concerned pest. The bacterial strain isolated from hairy caterpillars of *Arctornis submarginata* was tested to be a *Bacillus* with typical spore. The strain showed a longer doubling time and difference in biochemical tests from *Btk*. Moreover, this strain (Arc 01), showed a lower LC_{50} value and a shorter LT_{50} values as compared to *Btk* when tested against early instars of *A. submarginata*. Thus proving that the strain had a definite killing efficacy against the concerned pest when tried in laboratory conditions. Importance of nucleopolyhedrovirus (NPV) extracted from cadavers of looper caterpillars (*B. suppressaria* and *H. talaca*) could be well realized due to their high infectivity and low LC_{50} values. Field application of NPV (1 x 10^9 OBs/ml) proved efficacious in bringing down the looper population in Terai tea estate. Besides the *Bacillus* strain of *A. submarginata*, a granulovirus was also isolated from naturally infected population. The GV was found to have an LC_{50} value, 4.46×10^4 OBs/ml and LT_{50} values, 3.87 days for 1×10^7 OBs/ml concentrate. This GV added an effective bioagent to the biopesticide arsenal for future control of this emerging pest, especially in the organic and biorational tea plantations of Himalayan Terai and foothills.

Key words: *Camellia sinensis, Arctornis submarginata, Buzura suppressaria, Eterusia magnifica, Hyposidra talaca*

INTRODUCTION

Tea plantation (*Camellia sinensis* O'Kuntz), spreads over the hill slopes of Darjeeling Himalaya and its adjoining foothills and plains, known as Terai and the Dooars. After Assam, Darjeeling plantation including Terai and the Dooars areas provide the second largest yield of made tea from North-East India with a high export potential as "Darjeeling brand" with a certification trade mark (GI). Most quality tea clones, marked byflavor are grown at 500 to 2000 mt. altitude in Darjeeling hill while the high yielding clones are mostly planted in Terai and the Dooars foothills and their adjoining plains with a total yield of about 175 million kg/yr.

Distribution of insect pests also showed a pattern with dominance of sucking pest such as the jassid (*Empoasea*

Journal of Biopesticides 3(1 Special Issue) 114 -116 (2010)

Biocontrol potential of a newly isolated bacterial agent against *Arctornis submarginata* (Walker) (Lepidoptera: Lymantriidae) occurring in Darjeeling Terai region.

Sangita Khewa (Subba) and Ananda Mukhopadhyay

ABSTRACT

A strain of *Bacillus* causing disease in caterpillars of *Arctornis submarginata*, a defoliator of tea crop, was isolated from Darjeeling terai region. The strain showed positive reaction in lysine decarboxylase, ornithin decarboxylase, Voges-Proskaur, citrate utilization, nitrate reduction and in utilization of trehalose and glucose; difference with *Btk* was observed in ONPG test, and in utilization of citrate, arabinose, xylose, cellobios, melibiose and saccharose. The doubling time was 84 min, which is exactly the double of that of *Btk.* Difference was not evident in protein profile of the strain with that of *Btk.* The LC_{50} value was found to be 398.1 µg/ml with fiducial lower limit 353.06µg/ml and UL 443.14 µg/ml. The LC_{50} value of the new strain was lower than that of *Btk,* which was found to be [537.0 µg/ml; LL 483.63µg/ml and UL, 590.37µg/ml. The LT_{50} values of the new strain were also lower than that of *Btk*. These values were, 7.28 days for 1000 µg/ml and 8.88 days for 750 µg/ml as compared to the LT_{50} values 7.57 days for 1000 µg/ml and 9.5 days for 750 µg/ml of *Btk.* This findings opened up the possibility of developing new strain as microbial pesticide after standardizing its formulations and determining its safety aspects.

Key words: *Arctornis submarginata*, *Camellia sinensis*, biopesticide, crop pest

INTRODUCTION

Arctornis submaginata, commonly called as hairy caterpillar, is emerging as a potential pest of tea (Mukho padhyay and Roy, 2009). Swarms of caterpillars defoliate the mature and maintenance leaves adversely affecting the tea yield at the Darjeeling foothill region (Terai) (Mukhopadhyay *et al.,* 2007). *A. submarginata* has been found in North East Himalaya, Borneo and Sumatra on bamboo and other hosts (Schintlmeister, 1994). To combat the pest problem of tea Chemical pesticides are mainly used, with some backlashes such as, environmental pollution, human health hazard, resistance and resurgence in pests (Sarker and Mukhopadhyay, 2006). Hence, efforts are being made to evolve alternative strategies to manage this pests. One such approach is development and application of microbial bioagents. Commercial formulations of *Bacillus thuringiensis kurstaki* applied for management of this pest could not produce desirable results against *A. submarginata* swarms.

The objective of the present study was aimed to isolate and characterize a naturally occurring bacterial pathogen of *A.submarginata* and to know its potential as biopes ticide through determination of its median lethal concentration and time.

Bacillus thuringiensis kurstaki was used as a reference for comparison. Pest management using microbial pesticides would greatly help in production of export quality tea, free of pesticide residues.

MATERIALS AND METHODS

A bacterial strain was isolated from dead or moribund larvae of *A. submarginata*. For isolation of bacteria, the larvae dying of disease was taken for surface sterilization with 70% alcohol and then washed thrice with double - distilled water. These were then stored in double - distilled water within sterilized eppendorf which was wrapped with parafilm and stored at -20°C (Lacey and Brooks, 1997). The stored dead larvae were taken and thoroughly macerated by glass homogenizer. Crude homogenate was sieved through fine sterilized cotton cloth. The filtrate was centrifuged at 3000 rpm for 30 min. The supernatant was discarded and the precipitate containing bacterial suspension was taken for pure culture isolation by 'dilution streak method' in nutrient agar medium. The infectivity of the pure cultured bacterium was determined following Koch's postulate by infecting healthy second instar larvae. After proving the Koch's postulate the viability of the bacterium was checked by inoculating newly prepared agar medium at weekly intervals.

before noon, indicating significantly higher percentage of trapping between 6.00 a.m. and 8.00 a.m. (67.00 %) followed by 8.00 a.m. to 10.00 a.m. (26.03 %) and 10.00 a.m. to 12.00 noon (3.35 %). In after noon session (12.00 noon to 20.00 p.m.) only 3.62 per cent adults were trapped. During dark period i.e., 20.00 p.m. to 6.00 a.m. (next day) not a single male was trapped.

From the results, it is concluded that, the adults of *B. cucurbitae* were most active during morning hours, while less active during noon hours and inactive during night hours in the pumpkin field. For effective management of melon fly in pumpkin, the poison bait should be applied in the morning hours of the day when the adults of *B. cucurbitae* are most active either for mating or seeking host for oviposition.

Reference

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Report of a Defoliator of Tea, *Arctornis submarginata* (Walker) (Lepidoptera : Lymantriidae) from Darjeeling, (West Bengal)

A. Mukhopadhyay, S.Khewa, D.De
Entomology research unit, Department of Zoology, University of North Bengal, Dist. Darjeeling 734013, India.

Arctornis submarginanata (Walker) is distributed in North East Himalayan region, Borneo and Sumatra islands (Schinumeister, 1994). Attack on tea bushes by this species is recorded for the first time in the plantation of Darjeeling terai. Hence, work was undertaken on the defoliator's biology such as preference and acceptability of stages of tea leaf (based on maturiy), duration and measurements of the development stages, and fecundity. The species seems to have a pest potential as it has adapted to the continuous monoculture of high yielding tea clones of Himalayan foothills and plains.

The adult moths of *A. submarginata*, collected from tea plantation of Darjeeling terai were reared at a mean temperature 20°C, RH 60% (during December to February 2007) in the Entomology research laboratory, Department of Zoology, University of North Bengal located at the base of Darjeeling hills.

Eggs were round biconcave, olive green, laid in clusters of 260-280 on the dorsal surface of tea leaves. They hatched in 6-7 days with 75% success. The post-embryonic development was for 39-46 days with six larval instars; first larvae was light yellow, 0.29 cm in length, which grew 17-18 folds to reach brownish-black 6th instar stage, covered profusely with hair (Table 1)

Preference for the quality (tenderness) of the tea leaf was studied using leaf-area meter. Young, mature and senescent leaves were offered as food and the consumption rate of 3rd instar in terms of leaf area (sq mm/hr) was estimated (n=30). As 3rd instar larvae were predominantly