Study on the Entomopathogenic Bacteria of major Lepidopteran Tea pests and Evaluation of their prospect as biopesticide

Thesis submitted to the

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(Zoology)

Submitted by

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Supervisors' Certificate

This is to certify that Ms. Damayanti De, M.Sc has worked on a Ph.D programme entitled **"STUDY ON THE ENTOMOPATHOGENIC BACTERIA OF MAJOR LEPIDOPTERAN TEA PESTS AND EVALUATION OF THEIR PROSPECT AS BIOPESTICIDE"** under our supervision and guidance and that she has fulfilled the requirements related to submission of Ph.D thesis. This work was carried our since 2006. Her research work embodies original findings based on well planned investigation. The dissertation submitted herewith is for the partial fulfilment of the degree of Doctor of Philosophy in Science (Zoology) of the University of North Bengal. The thesis material has not been submitted to any other University for any degree whatsoever by her or any one else.

We sincerely wish Ms. Damayanti De success.

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(Co-supervisor)

Date: 05 February 2010

Place: University of North Bengal

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1. Introduction

1.a Tea plantation of Darjeeling Terai and the Dooars

Tea is the foliage crop harvested from the monocultures of *Camellia sinensis* O' Kuntz. All over the world infusions of tea leaves is taken as a popular invigorating and refreshing drink having excellent medicinal properties (Chakravartee, 1995).

According to Muraleedharan (1991) the tea plant in India originated from the triangular region formed by Naga, Manipur and Lushai extending along the border between Assam and Burma. The tea in Asian mainland stretches to China in North and to the hilly areas of Burma, Bangladesh, Thailand and Viet Nam in the South East. In summary the tea plant originally grew in mountainous forest areas, and then gradually moved to lower elevations where the ecological conditions were different from its original sources, causing changes in its growth forms along with a change in pests and diseases structure.

There are more than 1,500 varieties of tea grown in the world in more than 25 different countries. It is cultivated as a plantation crop, in acidic soil, warm climate with at least 150 mm of rain per annum (Anonymous, 1973). The tea was first used in China as a medicinal drink and later became a popular beverage. Nowadays, it has gained popularity world wide (www.communityipm.org/docs/Tea_Eco-Guide/01_History.PDF).

Tea in India is produced only in certain places. The three principal tea growing regions are Darjeeling, Assam and Nilgiri. Darjeeling and Assam are in the North-East while the Nilgiri regions are in South India. In Darjeeling foothills and plains, the main tea growing regions are Terai and the Dooars. The Terai and the Dooars region is located between 26°16'and 27°13' north latitude and between 87°59' and 89°53' east longitude and are divided by the mighty river Teesta. The Terai is situated 91m above mean sea level with an average rainfall of 350 cm and an average maximum temperature of 35°C and a minimum of 12°C. The soil is moderately acidic, rich in organic material and is suitable for tea plantation. Besides heavy rainfall, Terai and its adjacent Dooars regions are well watered by a number of major rivers and a host of rivulets. The major rivers from east to west of Terai and the Dooars are: Siltorsha, Jaldhaka, Teesta, Mahananda, Balason and Mechi. Some of the tea estates in Terai region easily accessible by road,

namely are: Atol, Kamalpur, Maruti, Sanyashi, Gungaram, Dagapur, Simulbari, Haskhoya, Nischintapur, Longview and a few more.

The Dooars or *Duars* (=Doors) area comprises flood plains and the foothills of the eastern Himalayas in North-East India and is the gateway to Bhutan. The altitude of the Dooars region ranges from 90 m to 1750 m. Most of the rivers mark the borders of the Dooars tea districts mainly spread over the political district boundaries of Jalpaiguri. Depending on geophysical and climatic conditions, the Dooars region was divided in to six tea growing sub districts namely Damdim, Chulsa, Nagrakata, Binnaguri, Dalgaon and Kalchini. Monsoon generally starts from the middle of May and continues till the end of September. Winters are cold with foggy mornings and nights. Summer is mild and constitutes a short period of the year. According to the statistics of Tea Board (India), there are 158 big tea estates in the Dooars and total area under tea is 92095 hectares. Total production of made tea is 167 million kg with an average yield of 1816 kgs per ha. Besides these 1,67,245 number of small tea gardens are also recorded in the Dooars and its adjoining areas in West Bengal (Anonymous, 2003).

It was observed that about 78% of the countries total area of tea plantation is located in North-India. Out of the total, 53% is existing in Assam and 22% in West Bengal. (http://www.nabard.org/roles/ms/ph/tea.htm).

1.b Pest problem in tea with emphasis on lepidopteran pests

Various problems of tea estates such as illiteracy, poverty, lock out of tea gardens, diseases and efficacy of tea workers hamper the tea production and processing a lot. But one of the major causes of crop loss is due to pest attack, mainly the insect tea pests.

Annual production of tea in Terai has fallen in 2009 to 76 million kgs as compared to 80 million kgs produced in 2008, mainly due to pest attack and climate change (48th Annual General Meeting of Terai Branch of Indian Tea Association-report, 2010). It was estimated that 1034 species of arthropods infest tea plant (Chen and Chen, 1989). The most common pests of tea are mites, thrips, tea mosquito bug, tea eating caterpillars, mirids, scolytid beetles and termites (Ghosh, 2001). Since the magnitude of

pest infestation varies seasonally depending on altitude, climate and cultural practices, an exact assessment of crop loss in tea is anywhere between 6 and 14% (Banerjee, 1976a).

There are approximately, 300 species of insects, mites and nematodes infesting tea, in India (Banerjee, 1993; Muraleedharan *et al.*, 2001) out of which about 25 species have already been recognized as serious pests. Most of them appear to have accepted tea under cultivation as an ideal host plant (Mukherjee and Singh, 1993). It has been found that about 6-14% of tea is lost due to insects, mites and weeds (Banerjee, 1976a).

A steady loss of 10% due to over all pest attack is a generally accepted figure though it could be 40% in devastating attacks by lepidopteran defoliators (Banerjee, 1993). Lepidoptera form the largest order of the pest species followed by Hemiptera (Chen and Chen, 1989). Earlier study of tea pests was by Green, (1890), Watt and Mann, (1903). Information on biology of tea pests of North-East India are available from the works of Hainsworth (1952), Das (1965), Banerjee (1983a, b) and that of south India and Srilanka from the studies of Muraleedharan (1983).

Among the lepidopteran tea pests, looper caterpillar (*Buzura suppressaria* Guenee) is one of the major defoliating pests of tea (Fig. 1, Fig. 2). This pest was first collected from Nowgong district of Assam (Cotes, 1895). It is one of the common pests of tea (Antram, 1911). The looper is an active defoliator of tea (Hill, 1983) and its infestation may become devastating within a short period. Therefore, the time factor for determination of economic injury level becomes very critical (Chakravartee, 1995). On the other hand a new species of looper has emerged in the Dooars and Terai region, by the name *Hyposidra talaca* (Walker), also called black inch worm (Basumajumdar and Ghosh, 2004; Das and Mukhopadhyay, 2008) (Fig. 3, Fig. 4). It has, in recent years, become more dominant than *B. suppressaria* population in many tea estates. The species is polyphagous and is found to infest a large number of weeds and forest trees along with the young leaves of tea bushes, causing a havoc loss in the tea plantation.

Fig. 1: Typical looper (*Buzura suppressaria*) caterpillar

Fig. 2: Leaf damaged by *Buzura suppressaria* caterpillar

Fig. 3: Mature *Hyposidra talaca* larva (5th instar)

Fig. 4: Earlier stage of black-inch worm *Hyposidra talaca* (Walker)

Red slug caterpillar (*Eterusis magnifica* (Butl.)) is one of the occasional pests of tea of regional importance in North-East India (Fig. 5, Fig. 6). The approximate time of occurrence of several discrete broods of red slug was recorded by Mann and Antram, (1906). The occurrence of a different red slug species, *E. virescens* (Butl.). on tea is reported from south India by Rau, (1952). Even sporadic populations of the defoliating pest, red slug should not be allowed to grow to an epidemic stage and control measures should be taken once the pest is seen in tea plantation was suggested by Chakravartee (1995).

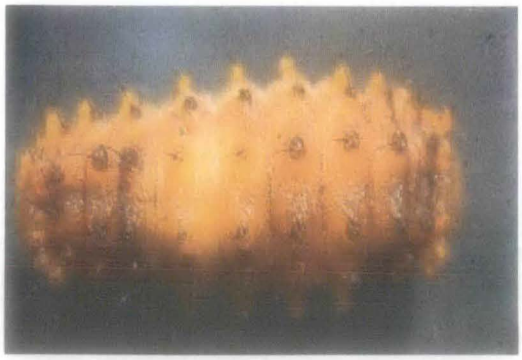


Fig. 5: Fifth (5^{th}) instar caterpillar of *E*terusia *magnifica*

Fig. 6: Injury of matured leaves of tea bushes by *E*terusia *magnifica*

The tea leaf roller (*Caloptilia theivora* Walsingham) has been known to infest tea from the dawn of plantation in North-East India (Fig. 7, Fig 8). Watt and Mann (1903) considered it to be a troublesome pest. It had never been serious in Assam and Bengal but occasionally caused considerable damage to tea in Dehra Dun. During 1988 the intensity of attack of this insect was found to be quite high in a few estates of Jorhat Assam circle when about 40-60% of the shoots in young and mature tea were found to be badly affected. In Darjeeling hill slopes and Terai seasonal occurrence of leaf rollers some times in large number has been witnessed. More than the quantitative loss caused by this pest, the quality of made tea deteriorates due to its faecal contamination.

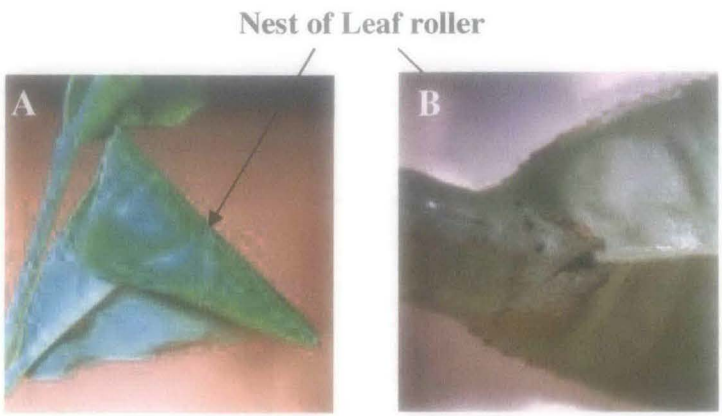


Fig. 7: A. and B. Nest of *Caloptilia theivora*

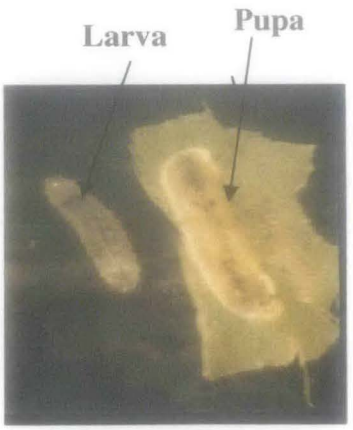


Fig. 8C Larva and pupa of *Caloptilia theivora*

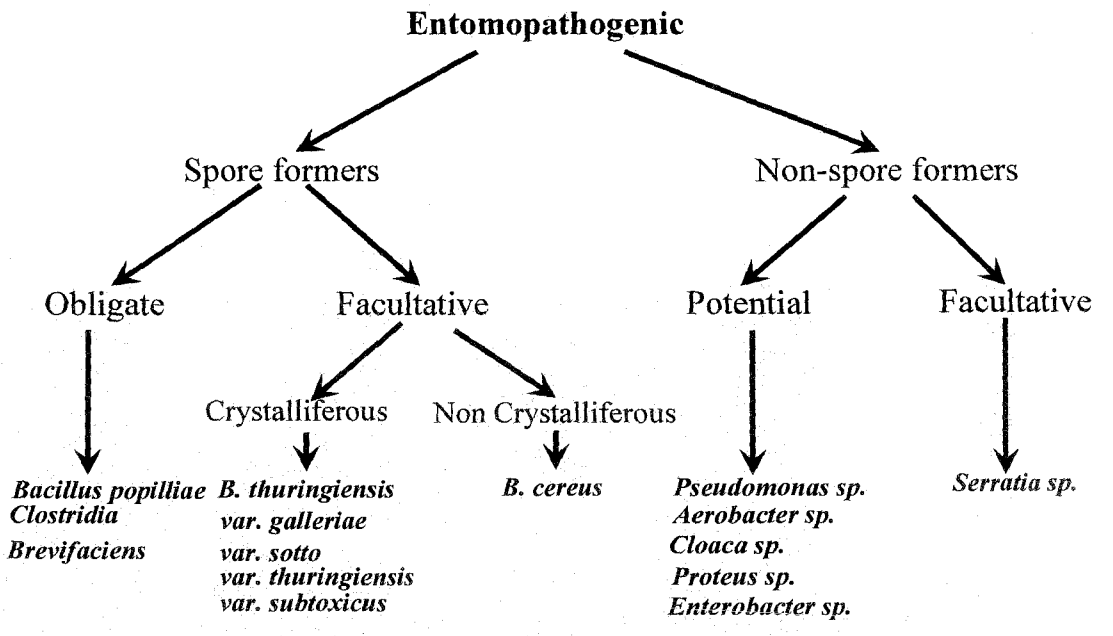
1.c Prospects of research with entomopathogenic bacteria as Biopesticide

To control pest problem in tea most conventional plantation use synthetic insecticides. Various problems concerning target, non-target and environment are associated with these chemical pesticides. Many synthetic pesticides 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 nontarget organisms (beneficial insects, mammals and human) (Chattopadhyay *et al.*, 2004). With long exposures to pesticides, resistant strains of insect emerge, requiring increased doses of insecticides and introduction of new insecticides. Different chemical pesticides (organophosphate & synthetic pyrethroids) have been found to be less effective against this defoliator in recent time (Sannigrahi & Talukdar, 2003; Sarker and Mukhopadhyay, 2006). Because of their harmful effects, application of a number of these insecticides has been banned by the EPA of the USA and even by the tea research organizations of India, and registration of many others are being reviewed (Chattopadhyay *et al.*, 2004). To minimize chemical residue in tea, European union and German law imposed stringent measures for the application of chemicals in tea and fixed MRL values at < or = 0. 1 mgkg(-1) for the most commonly used pesticides. This is seldom realized in the real practice and therefore, residue problem has become a major constraint for exporting tea to foreign countries especially to UK, Germany and the USA. In order to regulate the situation of the Indian market at global level, central insecticide board and prevention of food adulteration regulation committee have reviewed the MRL position for Indian tea and has recommended 10 insecticides, 5 acaricides, 9 herbicides and 5 fungicides for use in tea and issued the tea distribution and export control order 2005 which will help the country to limit the presence of undesirable substances in tea (Gurusubramanium *et al.*, 2008). Pesticide residues of organophosphorus (e.g. ethion and chlorpyrifos), organochlorine (e.g. heptachlor, dicofol, alpha-endosulfan, beta-endosulfan, endosulfan sulfate) and synthetic pyrethroid (e.g. cypermethrin and deltamethrin) in made tea, fresh tea leaves, soils and water bodies from selected tea gardens in the Dooars and Hill regions of West Bengal, India had already been determined. The organophosphorus (OP) pesticide residues were detected in 100% substrate samples of made tea, fresh tea

leaves and soil in the Dooars region. In the Hill region, 20% to 40% of the substrate samples contained residues of organophosphorus (OP) pesticides. The organochlorine (OC) pesticide residues were detected in 33% to 100% of the substrate samples, excluding the water bodies in the Dooars region and 0% to 40% in the Hill region. Sixteen percent and 20% of the made tea samples exceeded the MRL level of chlorpyrifos in Dooars and Hill regions respectively. The residues of heptachlor exceeded the MRL in 33% (April) and 100% (November) in the Dooars and 40% (April) and 20% (November) in the Hill region. Thus it was revealed that the residues of banned insecticides like heptachlor and chlorpyrifos in made tea are still present and may pose health hazards to the consumers (Bishnu *et al.*, 2008).

In view of this and also due to a greater acceptance of organic tea (as compared to 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 of insect pests is conservation of the microbial bio-agents or application of some of the effective bacterial and viral control agents in the tea ecosystem. Biopesticides are becoming one of the most promising tools for the control of agricultural pests all over the world. Biopesticide of bacterial origin has already showed prospects to replace chemical pesticide to a great extent (Chakravartee, 1995). The pesticide formulations in which entomopathogenic bacteria are the active components are known as bacterial pesticides (Borthakur, 1986).

Entomopathogenic bacteria are classified as spore formers and non-sporeformers, which are further subdivided into obligate and facultative. Again spore formers may be crystal forming (crystalliferous) or non-crystalliferous. The examples of these different groups are presented below in a schematic diagram (Srivastava, 2004):



The spore forming or sporulating bacteria form endospore which are highly resistant to environmental changes (temperature, humidity etc.) and allow them to persist in a dormant condition outside the intended host to develop into a bacterial cell on being ingested. The spore forming bacteria are, therefore more promising in insect control than the non-sporeforming ones. Among the spore formers, again, it is the crystalliferous ones which are better than non-crystalliferous ones because of the toxic nature of the crystals they produce. One of the best studied species amongst the crystalliferous bacteria is *Bacillus thuringiensis* which was first to be regarded as a potential microbial control agent by Steinhaus in 1956. This *Bacillus* is effective against the caterpillars of many Lepidoptera and exists in many strains or varieties which differ in their pathogenicity for different species of Lepidoptera. 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 (Goldberg and Margalit, 1977; de Barjac, 1981; Hansen *et al.*, 1996). The bioactivity of *Bt* serotypes 3a, 3b (Dipel R) and one of the components, Cry1Ab, was studied against the larvae of *Plutella xylostella*. *Bt* is also effective in controlling *Spodoptera litura* (Datta and Sharma, 1997) and *Crocidolomia binotalis* (Facknath, 1999). *Bt* accounts for more than 90% of the biopesticides used today (Feitelson *et al.*, 1992). Evaluation of various sub species for their toxicity was

carried out against the two lepidopteran pests, viz. *Spodoptera litura* (F.) and *Phthorimaea operculella* (Z.) (Putambekar *et al.*, 1997). The *Bt* strain which exhibited the highest activity against lepidopteran pests under laboratory conditions was evaluated for field efficacy on the pod boring pest complex of *Cajanus cajan* (Putambekar *et al.*, 1997).

Different formulations of *Bacillus thuringiensis* (*Bt*) has already been applied and tested earlier against many tea pests (Muraleedharan and Radhakrishna, 1989). The formulations of *Bt* were effective against leaf rollers. *B. thuringiensis* had already been used against tea pests *Caloptilia theivora* and *Cydia leucostoma* in South India (Unnamalai and Sekar, 1995). Many commercial formulations of *Bt* are used for controlling many important plant pests mainly caterpillars of Lepidoptera (butterflies and moths). The control effects of TA-BR combined with *Bt* on main tea pests *Euproctis pseudoconspersa*, *Ectropis obliqua* and *Andraca bipunctata* reached above 90%, and is safe for natural enemies (LingLing *et al.*, 2004). Various *Bt*-pesticides (from different strains of *B. thuringiensis*) were tested in Japan for their effectiveness on major lepidopteran insect pests of tea. *Bt*-pesticides appeared to be effective in controlling tea tortrix (*Homona magnanima*), smaller tea tortrix (*Adoxophyes* sp.), tea leaf miner (*Caloptilia theivora*) and other lepidopteran pests (Kariya, 1977).

Though different commercial formulations of *Bt* have already been used in controlling different types of insect pests all over the world the main and the foremost problem in using *Bt* as commercial formulation was the development of resistance among the insect pests against *Bt*. The wide spread appearance of resistance to *Bt* presents a cautionary tale for the way of using *Bt* and *Bt* toxin genes in pest management (Schnepf *et al.*, 1998). Other than this resistance problem, two problems are associated with commercialized *Bt* formulation. These include: (i) the longer period necessary to obtain high levels of mortality of pest larvae with *Bt* compared to chemical pesticides, (ii) a particular toxin is usually restricted to only one host insect so, there is a need to isolate more varieties of *Bt* with different and more toxic activity.

In the present research, study of naturally occurring bacterial bio-agents occurring in the lepidopteran (moth) pests of tea were done. There are chances that the bacterial

bioagents occurring inside the host body may be a well established novel bacterial entomopathogen, with the potentiality to kill the host than already available commercialized *Bt* formulation used against lepidopteran pests. Therefore the entomopathogenic bacteria of the caterpillar pest of tea were surveyed, isolated, characterized, then bioassayed in laboratory and testing in field for their killing efficacy. Expectedly such bacteria in future with proper biosafety testing may be developed in to microbial pesticides, and the same may be integrated in biocontrol and IPM programs developed to manage lepidopteran tea pests.

2

Review of literature

2. Review of literature

2.a Pesticide use pattern in tea cultivation of Darjeeling Terai and the Dooars and its associated problems

Tea (*Camellia sinensis* L.) is one of the most important cash crops in India due to its tremendous export potentialities and demand in the domestic market. It is grown in the Northern region of the state of West Bengal covering an area of about 1.03 million hectare in Darjeeling slopes, Terai and the Dooars regions and contributes about 21% of the total tea production of the country (Bishnu *et al.*, 2008). Pests, pathogens and weeds are the severe constraints of the productivity and quality of tea. As a result, the tea planters use a wide range of pesticides to combat these problems for high yield and economic returns. Though broad spectrum chemicals offer powerful incentives, they have serious draw backs such as resistance to pesticides, pest resurgence, outbreak of secondary pests, harmful effects on human health and environment due to the presence of undesirable residues. Because of the known toxicity of pesticide in food products (Nagayama *et al.*, 1995; Neidert and Saschenbreker, 1996) there is an increasing public concern regarding the pesticide residues in tea (Singh, 1984; Bishnu *et al.*, 2008). In the second half of the 20th century the use of pesticides in tea have been in the rise (Anonymous, 1976) which reached a mark of 1,850,000 litres in seventies (Banerjee, 1976b). A survey was conducted on pesticide use patterns in the tea plantations of Dooars during period 1998 to 2004. The study revealed that on an average 7.499l/kg of insecticides was used per hectare per year of which the organo-chlorine, organo-phosphate and carbamate (non-pyrethroid) accounted 73.5% and pyrethroid represent 36.6% during the survey period. The requirement of synthetic pyrethroid gradually increased with every passing year in all sub districts in Dooars (Roy *et al.*, 2008).Use of such a huge quantity has naturally created problems about their residues.

Samples taken from tea gardens in Darjeeling contained varying levels of residues in made tea. About 28 percent of 182 first flush samples and 31.5 percent of 89 second flush samples were found to carry residues above the maximum residual limits (MRLs). In another set of 65 samples of made tea, 43 per cent contained ethion residues with a maximum of 8.43 ppm while 18 percent of the samples contained dicofol residues of 6.4

ppm (Barooah, 1994) that were all above the MRL standards prescribed by international agencies such as EPA, CODEX, EU etc.

DDT and synthetic pyrethroids (SPs) have been found to induce quick insect resistance. Their common resistance mechanism is referred to as "knockdown resistance" or target site insensitivity (Beilschmidt, 1990).

Germany had rejected the Darjeeling Gold brand of tea from market leader Teekane because it contained 0.24 milligrams of tetradifon-a pesticide used against mites in tea. This was 24 times the MRL fixed by Germany (Singh, 2002).

During the last several decades, the control of pests, diseases and weeds in tea fields is predominantly by the use of synthetic chemicals. They have developed serious effects such as insect resistance to pesticides, resurgence of pests, outbreak of secondary pests, harmful effects on human health and environment (Muraleedharan and Selvasundaram, 2005). Extensive use of chemical pesticide has had many well documented adverse consequences (Ghosh *et al.*, 1994; Hajra, 2002).

Indiscriminate and excessive use of pesticides creates environmental pollution, bio-amplification and cumulation leading to health problem of top carnivores and humans, and resurgence of pests. At present it is a global concern, and to minimizing chemical residue in food staff including beverages, fruits and vegetables comes as a mandate. Some countries have specified very low residues for certain chemicals in tea. Germany has specified 2 ppm levels for Ethion and Dicofol against 10 ppm and 45 ppm respectively by Environmental Protection Agency (EPA) of USA (Barbora *et al.*, 1994).

General observations od available reports reveal that some of the tea gardens of foothills, Terai and the Dooars receive indiscriminate input of pesticides, to boost its production at the cost of undesirable residue levels in the environment (Sannigrahi and Talukdar, 2005; Roy *et al.*, 2007). Heptachlor and Chlorpyrifos pesticides despite being banned are prevalent in made tea at higher concentration than their respective MRLs (Bishnu *et al.*, 2008). This may pose serious health hazards to the consumers apart from losing export credentialities.

2.b Microbials as natural control agents

Tea plantation is severely affected by attacks of different insects of which lepidopteran caterpillars are the major defoliating pests. A non-conventional approach for combating the insect pests is largely based on microbial entomopathogens (Barbora *et al.*, 1994). An important benefit of microbial control agents is that they can be used to replace, at least in part, some more hazardous chemical pesticides. More selective and biodegradable biocontrol agents can provide important ecological benefits. One of the ecological advantages of microbial control agents is that they tend to be highly selective, infecting or killing a very narrow range of target pests (Joung and Cote, 2000). Entomopathogenic micro-organisms like fungi, bacteria and viruses have tremendous potentials in pest management. In tea also several bacteria have been identified as entomopathogenic to mite and insect pests as per records of the recent past (Chakravartee, 1995).

Microbial insecticides are composed of microscopic living organisms (viruses, bacteria, fungi, protozoa or nematodes) or the toxins produced by these organisms. They are formulated to be applied as or with conventional insecticidal sprays, dusts, liquid concentrates, wettable powders or granules (Weinzierl *et al.*, 2005).

The groups of pathogenic bacteria in insects are varied, and include species with the ability to infect uncompromised healthy insects and also a large number of opportunistic pathogens which multiply rapidly if they gain access to the haemocoel of stressed insect hosts through wounds or following infection (Aronson *et al.*, 1986).

Among spore forming *Bacilli* of lepidopterans, the use of *Bacillus thuringiensis kurstaki* (*Btk*) is well established as microbial biocontrol agent. The formulations of *Bacillus thuringiensis* were effective against leaf rollers and against many other tea pests (Muraleedharan and Radhakrishna, 1989). *Bt* products represent about 1% of the total 'agrochemical' market across the world.

Helicoverpa armigera (Hubner) (Lepidoptera: Noctuidae) a polyphagous pest has attained the status of Cosmopolitan pest in the recent years due to severe damage caused to several crops. The management of this pest becomes difficult as it has developed resistance to different chemical insecticides (Mehrotra and Pholeka, 1992; Lande and

Sarode, 1995). In view of this efforts are being made to evolve alternate strategies for the management of this pest. Investigations around the world have indicated the usefulness of *Bt* to combat the menace of insect pests. *Btk* has been found to be the most effective against early second instar larvae of *H. armigera* (Brownbridge and Onyango, 1992).

The use of entomopathogenic bacteria, *Bt* and *Bacillus sphaericus* as biolarvicides are viable alternative for insect control (Reis *et al.*, 2001). The toxins produced by *Bt* are highly specific and are harmless to humans, vertebrates and plants, and are completely biodegradable, so no residual toxic products accumulate in the environment (Schnepf *et al.*, 1998).

Btk is an agriculturally important organism because it kills lepidopteran insects. Another potentially useful strain, *Bacillus thuringiensis israelaensis* had been isolated that produced a toxin effective against mosquitoes and black flies (Yoshishu, 1993). Toxicity of spore crystal complexes and commercial formulations of *Bt* was evaluated against second instar larvae of the diamond back moth, *Plutella xylostella*. Different formulations of *Bt* namely Delfin WG, Dipel 8L, Halt WP etc. were studied against the larvae of *Plutella xylostella* (Pokharkar *et al.*, 2002). HD-1 strain of *Btk*, produces two kinds of entomocidal protein inclusions (delta-endotoxin), bipyramidal and cuboidal. The toxicity of this bacterium against lepidopteran insects is associated with bipyramidal toxins (Tojo, 1986). *Bt* is also effective in controlling *Spodoptera litura* (Datta and Sharma, 1997) and *Crocidolomia binotalis* (Facknath, 1999).

Btk kills the caterpillar stage of a wide array of butterfly and moths. In contrast, *B. popillae* kills only Japanese beetle larvae (Weinzierl *et al.*, 2005).

The microbial insecticides most widely used in the United States since 1960s are preparations of the bacterium *Bt* (Weinzierl *et al.*, 2005).

Bt accounts for more than 90% of the biopesticides used today (Feitelson *et al.*, 1992). Evaluation of various subspecies of *Bt* for their toxicity has been carried out against the two lepidopteran pests, viz. *Spodoptera litura* (F.) and *Phthorimaea operculella* (Z.) (Putambekar *et al.*, 1997).

Cajanus cajan (Pigeaonpea) is one of the major legume (pulse) crops of the tropics and subtropics (Nene and Sheila, 1990). In India, the major insect pests of this

crop are the pod boring lepidopteran pests damaging about 24-36% crops in the central and southern states of India (Lateef and Reed, 1983; Reed and Lateef, 1990). In order to check the pest menace the *Bt* strain which exhibited the highest activity against lepidopteran pests under laboratory conditions was evaluated for field efficacy on the pod boring pest complex of *Cajanus cajan* (Putambekar *et al.*, 1997). Pathogens of insects have been under evaluation as biological control agents for more than a century. With few exceptions, they are not effective as classical biological control agents. Moreover, even as insecticides, only *Bacillus thuringiensis* (*Bt*) has been a commercial success (Federici, 2006).

BLB1 is a new *Bacillus thuringiensis kurstaki* strain effective against third instar *Ephestia kuehniella* (Saadaoui *et al.*, 2009). An isolate of *Bacillus thuringiensis* has been identified with toxic activity against coleopteran insects but not against lepidopteran or dipteran species. Toxin crystals from this strain contain a polypeptide of approximately 64,000 molecular weight possessing insecticidal activity (Herrnstadt *et al.*, 1986).

A novel isolate of *Bacillus thuringiensis* subsp. *thuringiensis* produces a quasicuboidal crystal of Cry1Ab21 which is toxic to larvae of *Trichoplusia ni* (Swiecicka *et al.*, 2008).

Native *Bacillus thuringiensis* strains were isolated, characterized and tested for bioactivity against *Ephestia Kuehniella* (Lepidoptera: Pyralidae) larvae (Öztürk *et al.*, 2009). Mexican strains of *Bacillus thuringiensis* were selected, characterized and their activity was determined against four major lepidopteran maize pests (Bohorova *et al.*, 1996).

Cry1 genes was detected in *Bacillus thuringiensis* isolates from South of Brazil and activity was determined against *Anticarsia gemmatalis* (Lepidoptera:Noctuidae) (Bobrowski *et al.*, 2001). Brazilian strains of *Bacillus thuringiensis*, namely S701, S764 and S1265 were analysed regarding their cry gene and protein contents, crystal type, and activity against larvae of the lepidopteran fall armyworm (*Spodoptera frugiperda*), the velvet caterpillar (*Anticarsia gemmatalis*), the dipterans (*Culex quinquefasciatus* and *Aedes aegypti*) and the coleopteran (*Tenebrio molitor*) (Silva *et al.*, 2004).

The larva of *Scrobipalpuloides absoluta*, a South American moth, is the most devastating insect pest of tomato in Chile. The potential for using bacterial insecticides was studied analysing the relative toxicity of native *Bacillus thuringiensis* (*Bt*) isolates (Theoduloz *et al.*, 1997).

Other than *Bacillus thuringiensis* strains which were found to be entompathogenic to different insect pests of different family, few non-spore forming gram negative bacteria were found to be effective against insect pests. The larvae of scarab beetles, known as "white grubs" and belonging to the genera *Phyllophaga* and *Anomala* (Coleoptera: Scarabaeidae), are regarded as soil-dwelling pests in Mexico. During a survey conducted to find pathogenic bacteria with the potential to control scarab larvae, a native *Serratia* sp. (strain Mor4.1) was isolated from a dead third-instar *Phyllophaga blanchardi* larva collected from a corn field in Tres Marı´as, Morelos, Mexico (Nun˜ez-Valdez *et al.*, 2007).

The hazelnut leaf holer (*Anoplus roboris*, Coleoptera: Curculionidae) is a devastating pest of hazelnut and oak trees. It 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 leaf holer were investigated and tested for insecticidal effects. 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) (Demir *et al.*, 2002).

The alder leaf beetle (*Agelastica alni* L., Coleoptera: Chrysomelidae) is one of the sources of damage to hazelnut and alder trees throughout the world. In order to isolate more effective and safe biological control agent against *A. alni*, bacterial flora of the alder leaf beetles were isolated that were collected from the vicinity of Trabzon, Turkey. Based on morphological, physiological and biochemical tests, bacterial flora were identified as *Enterobacter agglomerans* (Aa1), *Listeria* sp. (Aa2), *Pseudomonas chlororaphis* (Aa3) and *Pseudomonas fluorescens* (Aa4) (Sezen *et al.*, 2004).

Despite a more than 10 fold increase in insecticide use since 1940 (Lysansky and Coombs, 1994), crop losses due to insects have nearly doubled in the same period. The

situation demands movement towards better control methods among which microbial control is most efficient. The most promising biological control agent is *Bacillus thuringiensis*, the leading organism being used as commercial microbial pesticides (Lambert and Pferoen, 1992; Meadows, 1993; Lysansky and Coombs, 1994).

The microbial control of insect pests is of crucial importance in developing countries (Dulmage, 1993). Overuse or misuse of chemical pesticides and their negative impacts on soil, and water quality, human health, wild life and ecological balance within agro-ecosystems are increasingly becoming causes of concern, underlining the need for development of alternative pest control methods (Meadows, 1993).

2.c Importance of isolation of new strains of bacteria for developing potential biopesticides

Bacillus thuringiensis is a bacterium known for producing protein crystals with pesticidal properties. These toxins are widely sought after for controlling agricultural pests due to both their specificity and their applicability in transgenic plants. Although any particular toxin has a desirably restricted host range, there is a large number of different toxins, each showing toxicity to one or many diverse pests (Rampersad and Ammons, 2005). For these reasons there is currently great interest in isolating strains of *Bt* with either unique host specificity or elevated toxicity.

Constraints to greater use of *Bt* in developing countries are: 1) scientific and technical: the difficulty in increasing the effectiveness of the products against specific pests and under specific agro-ecological conditions. 2) micro and macro-economics: efforts to reduce costs of production in developing countries, thus making *Bt* useful only in small scale application and limiting its large scale commercialization. 3) farmer acceptability: the longer period necessary to obtain high levels of mortality of pest larvae with *Bt* compared to chemical pesticides often prevent farmers from readily accepting *Bt*-based insecticides.

The main and the foremost problem encountered commercial formulation of *Bt* was the development of resistance in the insect pests against *Bt*. A number of insect populations of several different species with different levels of resistance to *Bt* have been reported (Schnepf *et al.*, 1998). The pest resistance was registered for *Bacillus*

thuringiensis kurstaki, *Bacillus thuringiensis israelensis* and other *Bt* subspecies. The insects resistance to *Bt* include that of *Plodia interpunctella*, *Cadra cautella*, *Leptinotarsa decemlineata*, *Chrysomela seripta*, *Trichoplusia ni*, *Spodoptera litoralis*, *S. exigua*, *Helicoverpa virescens*, *Ostrinia nubilalis* and *Culex quinquefaciatus*. It is clear that this wide spread appearance of resistance to *Bt* presents a cautionary tale for the way of using *Bt* and *Bt* toxin genes in pest management with a review of the resistance management program of *Bt* (Schnepf *et al.*, 1998).

Due to different constraints of using *Bt* in the field, scientists all over the world are engaged to isolate new strains of *Bt* and develop them for controlling different pest insects. Selection of *Bt* strains for controlling fall armyworm (*Spodoptera frugiperda*) has already been worked out (Polanczyk *et al.*, 2000). Any particular *Bt* toxin has a desirably restricted host range, there is a large number of different toxins each showing toxicity to one of many diverse pests. For this reason currently research is going on to isolate novel strains of *Bt* with either unique host specificity or elevated toxicity. There are numerous reports on isolation of novel *Bt* strains from the environment (Rampersad and Ammos, 2005; Bernard *et al.*, 1997; Vilas-Boas and Lemos, 2004; Mohan *et al.*, 2009).

The great extension of tea belts in North Bengal region especially in the Terai and the Dooars regions, and diversity of insects provide the opportunity of isolating novel entomopathogenic bacteria. The identified bacterial strains analyzed in this research investigation represent a sample of diversity and natural resource.

Under the new plant protection strategy especially organic farming in tea, biological control plays an important role. Knowledge based technologies, free from inputs of harsh chemicals are now needed to produce bio-organic tea. Pest management technologies must be economical, robust, reliable, practical and ecologically benign. Once such integrated pest management modules are developed using appropriate control techniques including biological control in a mutually reinforcing manner, a check on the tea pest populations to a non-damaging level can be easily obtained. Entomopathogenic microbials like bacteria hold a great promise for developing such ecofriendly pest management strategies.

3

Objectives & Scope of study

3. Objectives and scope of study

The objectives of the research study include:

a) Screening of four lepidopteran species of tea pests for bacterial pathogens.

The pest species will include:

(i) loopers (*Buzura suppressaria* and *Hyposidra talaca*),

(ii) red slug caterpillars (*Eterusia magnifica*) and

(iii) leaf rollers (*Caloptilia theivora*)

occurring in the plantations of Darjeeling foothills and its adjoining plains (Terai and the Dooars).

b) Isolation and preliminary characterization of the bacterial entomopathogens.

c) Bioassay of the bacterial pathogens to find their value as biocontrol agents against target tea pests.

d) Determining the cross-infectivity of the bacterial isolates to other lepidopteran pests and to the beneficial insect, silk worm.

4

Materials & methods

4. Materials and methods

A. Survey of major lepidopteran tea pests: a study on the occurrence of bacterial infection:

A.a Tea Estates/plantations surveyed for tea pest

The main tea plantation areas surveyed for collecting lepidopteran specimens were Terai and the Dooars region of Darjeeling foothills and adjoining plains. Samples were collected from East, West, South, North and Central areas of Terai and the Dooars. The name of the main tea estates surveyed in Terai were (Fig. 9, Fig. 10):

I. Terai region

a) Central: Kamalpur T.E., Panighata T.E., Bengdubi T.E.

b) Southern: Bidhannagar T.E., Hashkhoya T.E., Gungaram T.E.

c) Western: Sanyashi T.E., Atol T.E., Batashi T.E. Maruti T.E.

d) Northern: Matigara T.E., Nischintapur T.E., Dagapur T.E. Simulbari T.E.

II. The Dooars region

a) Eastern: Bhatkhawa, Chuapara, Kumargram and Sankosh T.E.

b) Central: Binnaguri, Mujnai and Hantapara T.E.

c) Northern: Jiti and Nagrakata T.E.

Major lepidopteran tea pests were collected from these tea estates. Infected state of larvae and cadavers were also collected. Collected active larvae were reared in the laboratory condition for observation. The larvae dying in laboratory reared population were also studied for estimating portion of natural population dying out of bacterial infection and also for isolation of the causative entomopathogens.

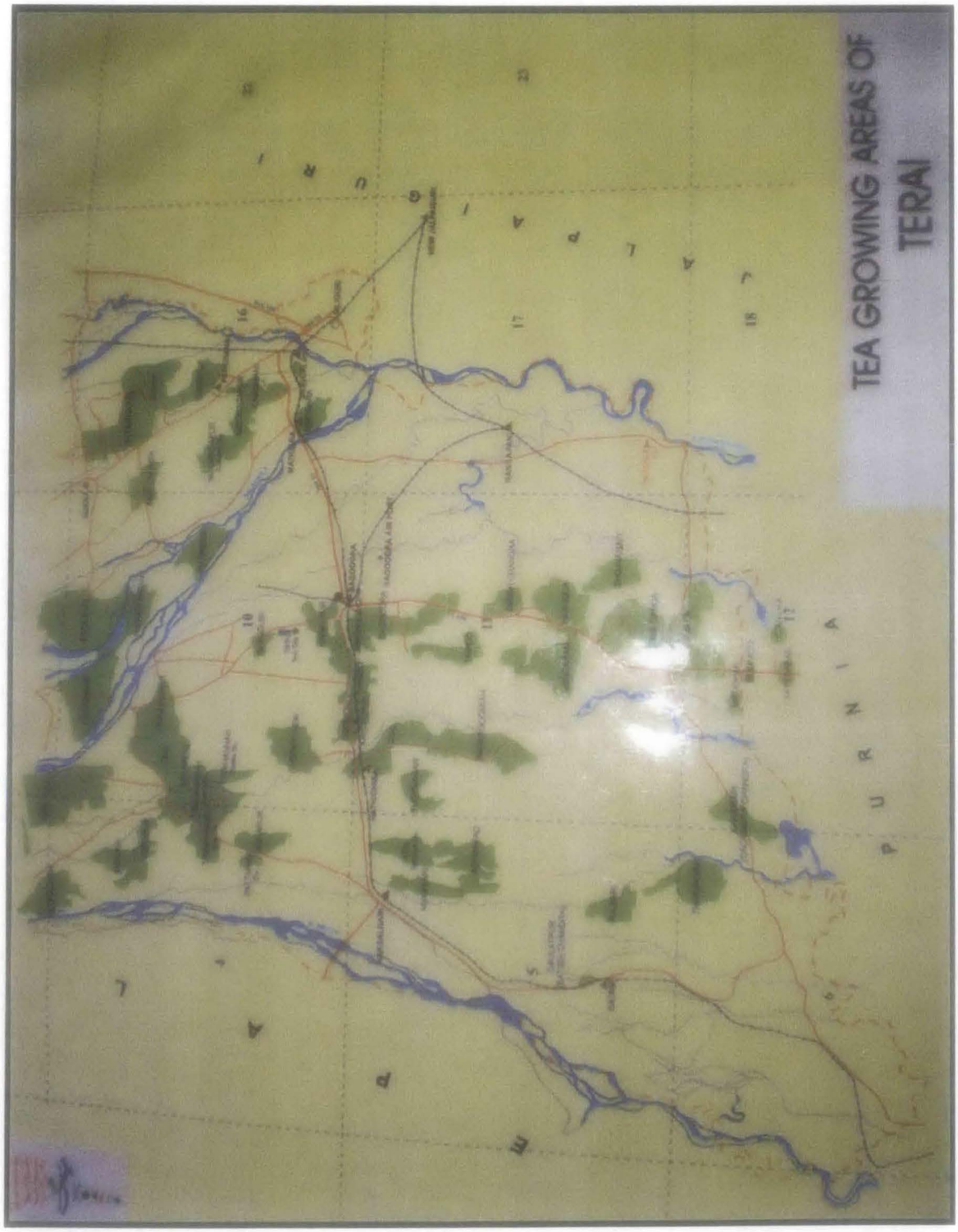


Fig.9 Map showing the tea growing areas in Terai region

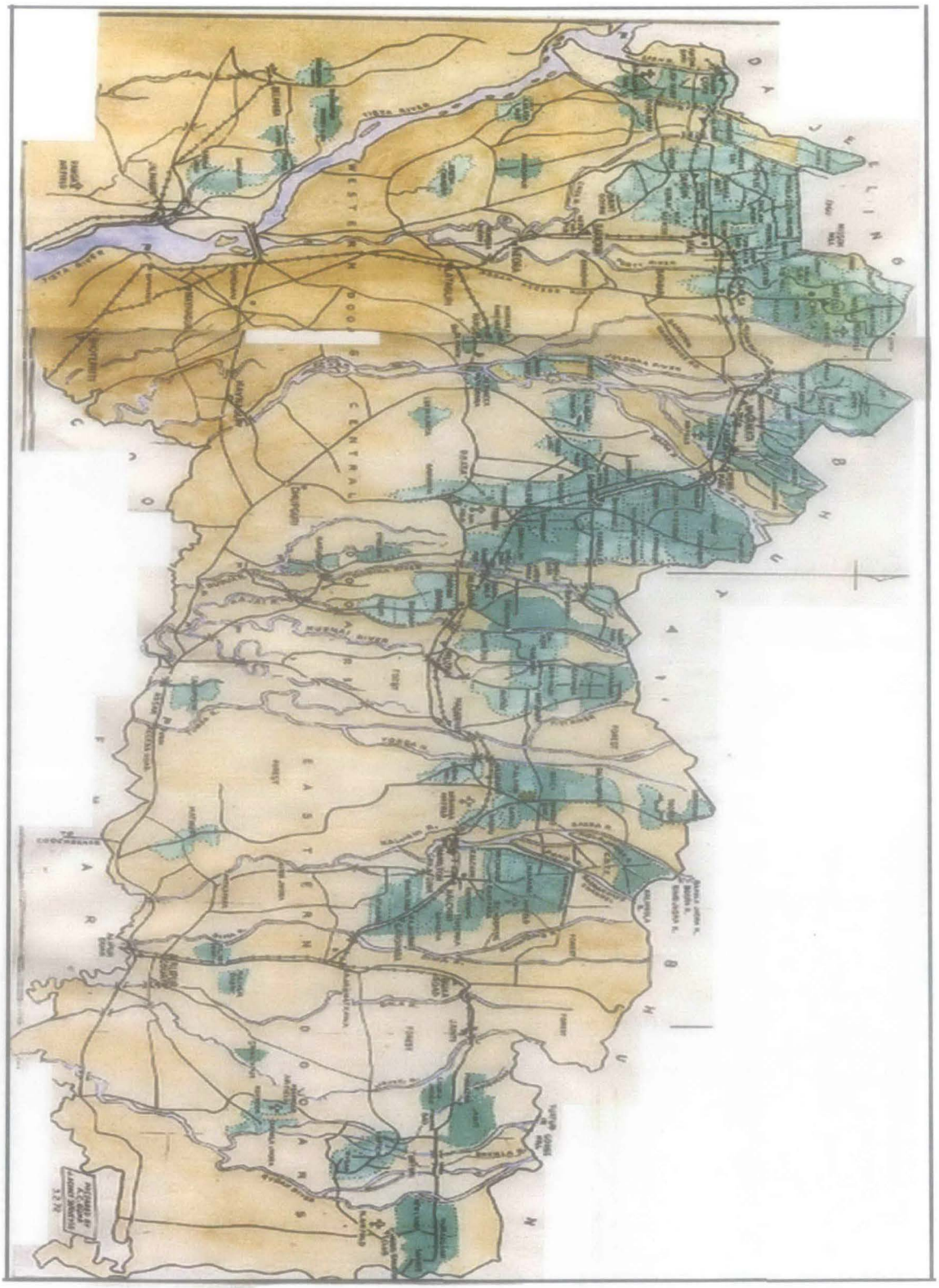


Fig.9 Map showing the tea growing areas in Dooars region

A.b Collection and processing of dead larvae

Dead and diseased larvae were collected from the field and laboratory reared population of respective tea pests and dipped in 70% alcohol for two seconds for surface sterilization. Then the dead larvae were washed three times with sterilized double distilled water and then kept in sterilized eppendorf tube with sterilized double distilled water. The tube was wrapped with parafilm and stored at –20°C (Lacey and Brooks, 1997).

A.c Isolation of Entomopathogenic Bacteria

For isolating bacteria, the stored dead larvae were taken and thoroughly macerated by glass homogenizer and the 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 (Lacey and Brooks, 1997). The infectivity of the bacteria was determined following Koch's postulates by infecting healthy first instar larvae with the aforesaid cultured bacteria. After proving the Koch's postulates the viability of the bacteria was checked by inoculating in newly prepared agar medium at weekly intervals.

B. Characterization of bacterial strains isolated from the lepidopteran pest species (viz. *Buzura suppressaria*, *Hyposidra talaca*, *Eterusia magnifica* and *Caloptilia theivora*)

B.a Morphological Characteristics

Determination of cell morphology

The morphology of the bacterial cell was determined after culturing the bacteria overnight in nutrient agar medium in a petriplate, incubated at 37°C. A smear of the overnight grown culture was taken in a glass slide and observed under phase contrast microscope (Olympus, CX31) (oil immersion, 1000X). To determine the motility of a

bacterium, a drop of culture (24 and 48 hours old) in nutrient broth medium was used to prepare a hanging drop in a cavity slide. The drop was observed using a phase contrast microscope (Olympus, CX31) (oil immersion, 1000X).

The spore and crystal protein shape of bacteria were determined after growing bacterial culture in nutrient agar medium for 4 days (up to sporulation) in incubator at 37°C. The spore-crystal mixture was suspended in 1 ml ice-cold 1M Nacl and centrifuged for 5 min at 12,000 rpm. The pellet was suspended in distilled water. The presence of spores and crystals was recorded directly by preparing a smear of this culture in a glass slide and observed under phase contrast microscope (Olympus, CX31) (oil immersion, 1000X) (Kati *et al.*, 2007).

The colony morphology and colony texture were determined in overnight grown culture prepared in glass petriplate and observed with naked eye.

Bacillus thuringiensis kurstaki (*Btk*) was used as control for comparative study, obtained from an authenticate Institutional source.

B.b Biochemical Characteristics

Several biochemical tests 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 control.

Enterobacteriaceae Identification Kit (KB003 Hi25) (Himedia) was also used to identify entomopathogenic *Enterobacter* sp.

Some more tests such as gram reaction, production of catalase and determination of anaerobic growth were also performed without the help of biochemical testing kit. A brief description of these biochemical tests are given below:

Gram staining

The method of Bartholomew (1962) was followed. A suspension of a 24 hours old culture on nutrient agar medium was prepared in distilled water. A drop of that suspension was smeared on a grease-free slide. The smear was air-dried, heat-fixed,

flooded with crystal violet stain for 1 minute, and washed for 5 second with water. The smear was then flooded with Burke's iodine solution, allowed to react for 1 minute, washed again for 5 second with water. The smear was then observed under compound microscope (100X) for stained bacteria.

Production of catalase

A 24 hours old slant culture was flooded with 0.5 ml of 10% hydrogen peroxide solution and observed for the production of gas bubbles, indicating the presence of catalase (Norris *et al.*, 1981).

Anaerobic growth

Anaerobic agar medium was distributed into culture tubes in amount sufficient to give 7.5 cm depth of the medium and sterilized by autoclaving at 121°C 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. They were incubated at 30°C for 7 days, and observed for growth along the length of the stab (anaerobic) and surface of the agar (aerobic) (Claus and Berkeley, 1986).

Biochemical testing kit

The kit materials consisted of medium for 24 biochemical tests and separate disc for oxidase test. A brief description containing, name of each test, name of reagents to be added after incubation of bacterial culture in kit medium, original colour of each medium, positive reaction of a bacterium and negative reaction was given as is mentioned in the following table (Table.1)

Table.1 Details of biochemical tests used for identifying bacterial strains.

Serial No.	Name of biochemical test	Reagents to be added after incubation	Original colour of the medium	Positive reaction	Negative reaction
1.	ONPG	-	Colourless	Yellow	Colourless
2.	Lysine decarboxylase	-	Olive green	Purple	Yellow
3.	Ornithin decarboxylase	-	Olive green	Purple	Yellow
4.	Urease	-	Organish yellow	Pink	Organish yellow
5.	Phenylalanine deamination	2-3 drops of TDA reagent	Colourless	Green	Colourless
6.	Nitrate reduction	1-2 drops of sulphanilic acid and 1-2 drops of N,N-Dimethyl-1-Napthylamine	Colourless	Pinkish red	Colourless
7.	H_2S production	-	Organish yellow	Black	Organish yellow
8.	Citrate utilization	-	Colourless	Blue	Yellowish -green
9.	Voges-Proskauer's	1-2 drops of Baritt reagent A and 1-2 drops of Baritt reagent B	Colourless	Pinkish red	Colourless/ slight copper
10.	Methyl red	1-2 drops of Methyl red reagent	Colourless	Red	Yellow
11.	Indole	1-2 drops of Kovac's red reagent	Light green	Pinkish red	Colourless
12.	Malonate	-	Cream	Blue	Light green
13.	Esculine hydrolysis	-	Red	Black	Cream
14.	Arabinose utilization	-	Red	Yellow	Red/pink
15.	Xylose utilization	-	Red	Yellow	Red/pink
16.	Adonitol utilization	-	Red	Yellow	Red/pink
17.	Rhamnose utilization	-	Red	Yellow	Red/pink
18.	Cellobiose utilization	-	Red	Yellow	Red/pink
19.	Melibiose utilization	-	Red	Yellow	Red/pink
20.	Saccharose utilization	-	Red	Yellow	Red/pink
21.	Raffinose utilization	-	Red	Yellow	Red/pink
22.	Trehalose utilization	-	Red	Yellow	Red/pink
23.	Glucose utilization	-	Red	Yellow	Red/pink
24.	Lactose utilization	-	Red	Yellow	Red/pink
25.	Oxidase test	Done on oxidase disc separately. Detects cytochrome oxidase production.	White	Deep purple within 10 sec.	White/ purple after 60 seconds

B.c Physiological Characteristics of bacterial strains

B.c.i Growth characteristics: determination of generation time

In this procedure, growth of bacterial strains was determined by turbidimetric method after Cappuccino and Sherman (1996). The growth of the bacteria was determined in nutrient broth medium (Himedia Co.). In this procedure one loopful of bacterial culture (from one day old culture) was inoculated in nutrient broth medium and incubated at 37°C in static condition in incubator. The reading of the O.D. value of the growing culture was taken at 540 nm in spectrophotometer every 30 minutes interval up to the stationary phase of growth of bacteria. Finally optical density and time interval were plotted on a graph paper and from this the generation time (doubling time) was determined. *Bacillus thuringiensis kurstaki* was used as control for comparison.

B.c.ii SDS-PAGE profile (Qualitative) of crystal protein

Bacterial strains were grown in Luria Bertani medium at 37°C without shaking. It was grown up to the phase of sporulation. Visual confirmation of crystalline bodies was done with the aid of phase contrast microscopy. The crystal was harvested in high pH buffer of sodium carbonate and 2-marcaptoethanol (Kranthi, 2005) with slight modification (2-marcaptoehtanol was used instead of Dithiothreitol (DTT)) in incubator for 2 hours at 37°C. Then the crystal was separated from spore-crystal mixture with the help of cooling centrifuge operated at high speed (10000 rpm) for 15 minutes. The supernatant containing crystal protein was separated from spores accumulated as precipitate. This crystal protein was taken for SDS-PAGE analysis.

B.c.iii Qualitative (SDS-PAGE) analysis of whole body protein of bacteria

The bacterial strains were 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-marcaptoethanol, 70% Tris-HCL, P^H 6.8, and 4% deionized water. The extracted protein was analysed by SDS-PAGE after the method of Costas (1992). Gels were fixed in trichloroacetic acid and stained with Coomassie Brilliant blue G-250 (Neuhoff *et al.*, 1988). The molecular weights of proteins were

determined by using protein molecular weight standards [Broad range (PMW-B) from Bnagalore Genei Co.].

C. Bioassay: Determination of LC_{50} value and LT_{50} values

For bioassay about 250 larvae of lepidopteran pests were collected from tea plantations of Tocklai vegetative clone (namely TV-25). The plantations were about 20 years old mainly growing in Darjeeling Terai and the Dooars region. The larvae were reared in laboratory and passed through two generations at 27 ± 2^0 C; 72 ± 2 % R^H under a photoperiod phases (L: D) of 13:11 hrs. in aseptic conditions. Fresh tea twigs/leaves (TV-25) from the experimental tea garden organically maintained on North Bengal University campus was supplied as food. The two-day old second instar larvae were used for every bioassay test.

The efficacy of the entomopathogenic micro-organisms was determined by bioassay (Fig. 11). In case of spore and crystal producing gram positive bacteria, extraction and bioassay was done after the procedure of Dulmage *et al.*, (1970). Different concentrations of crude samples (spores and crystal) (100, 300, 500, 750 and 1000 µg/ml) 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 Sekar (1995). In case of non-spore forming bacteria aqueous formulation of bacteria (vegetative body) was used for bioassay (100, 300, 500, 750 and 1000 µg/ml) after the method of Jackson *et al.*, (1992).

One hundred larval specimens of second instar were taken for treatment with each bacterial concentration (both in case of spore forming and non-spore forming bacteria). Sterile distilled water was used in control. The mortality was observed at an interval of 24 hrs from the day of inoculation (since 1^{st} day of feeding up to 9^{th} 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; Damgaard *et al.*, 1998). Data were subjected to probit analysis (Finney, 1954) 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):

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

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.

Fig. 11: Bioassay set up in laboratory

D. Cross infectivity testing

D.a Other lepidopteran tea pests

Cross infectivity of the isolated entomopathogenic bacteria to other lepidopteran tea pests sharing same niche were tested.

In case of spore forming and crystal producing bacteria crude spore-crystal mixture (100, 300, 500, 750 and 1000 µg/ml) were taken for cross infectivity testing. Aqueous formulations of bacterial concentrations (vegetative body) (100, 300, 500, 750 and 1000 µg/ml) were used in case of non spore-forming bacteria. One hundred larval specimens of second instar were taken for treatment with each concentration. Sterile distilled water was used in control. Tea leaves dipped in different concentrations were offered as food; only distilled water was used in control.

The mortality was observed at an interval of 24 hrs from the day of inoculation (1^{st} day feeding up to 9^{th} day). The corrected mortality was calculated using Abbott's

formula (Abbott, 1925). Data were subjected to probit analysis (Finney, 1954) 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).

D.b Mulberry silkworm (*Bombyx mori*) (Locally cultured multivoltine race)

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 mulberry silk worms before spraying in the tea plantations. Experiment was done for determining the infectivity in the laboratory condition. For the experiment the multivoltine silkworm (nistari variety) was taken (Fig. 12). Disease free layings (DFLs) were collected from West Bengal Sericulture Department situated at Matigara, Siliguri. Freshly hatched neonates of silkworm were given fresh sterilized mulberry leaf collected from North Bengal University campus and from West Bengal Sericulture Department, Matigara. They were reared up to second instar and were taken for the cross infectivity experiment. In case of spore and crystal protein forming bacteria crude spore-crystal mixture (100, 300, 500, 750 and 1000 µg/ml) were taken for silk worm infectivity testing. Aqueous formulation of vegetative body (100, 300, 500, 750 and 1000 µg/ml) were used in case of non spore-forming bacteria. Hundred second instar larvae of silkworms were treated with each concentration. Sterile distilled water was used in control. Silkworm larvae were kept under everyday observation upto 9^{th} day of inoculation (first day feeding) for recording any mortality.

Fig. 12: Silk worm (*Bombyx mori*) larvae feeding mulberry leaves.

E. Field trials on biocontrol efficacy

To find out the efficacy of a particular entomopathogenic bacterium in field condition, field study was conducted in the tea estates of Terai region (Fig. 13). The experiment was conducted in a randomized block design (RBD) with three replicates for each treatment as well as control. Four different concentrations of an entomopathogen was sprayed (as separate treatment) after the method of Guerrero *et al.*, (2007) and Ravi *et al.*, (2008). Atleast thirty pest infested tea bushes (TV-25 cultivar) were taken per replicate. A fairly large population (≈100/bushes) of second instar larvae were treated with the bacterial formulations at different concentrations (Salama and Salem, 1999). Water was used as carrier and high volume knapsack sprayer was used. Precount of insect larvae was taken before spraying to determine the number of larvae per bush exposed to each treatment and also in the control (Guerrero *et al.*, 2007; Ravi *et al.*, 2008; Salama and Salem, 1999). After spraying the data of live larvae recovered was taken on 3rd, 5th and finally on 7th day as per the method prescribed by Salama and Salem, (1999) and Ravi *et al.*, (2008). To evaluate the relative efficacy of each treatment the numbers of live larvae recovered in each treatment was compared with that of control plots after Guerrero *et al.*, (2007) and Hellman, (1994). The percent of live larvae on 3rd, 5th and 7th day was graphically represented (Mean±SD) after Guerrero *et al.*, (2007) and Cory *et al.*, (1994). The percentage live larvae after arcsine transformation were compared between treatments after 3$^{rd.}$ 5th and 7th day using one-way analysis of variance (ANOVA) (Guerrero *et al.*, 2007; Ravi *et al.*, 2008).

Fig. 13: Field experiment of microbial formulation

F. Statistical analysis

Quantitative results were subjected to standard statistical analysis. Software packages such as SSP, SPSS, Origin 50 and other were used. Results of bioassay and cross infectivity experiments were subjected to log Probit analysis. MS-EXCEL program was mainly used for analysis and graphics.

5 Results

5. Results

(I) Loopers (*Buzura suppressaria* and *Hyposidra talaca*)

A. Symptoms of bacteria infected larvae

The bacteria infected larvae turned characteristically blackish with significant shrinkage of body and discoloration followed by rapid decomposition. The photographs comparing healthy larva and the dead larva due to bacterial infection, show marked differences (Fig.14, Fig.15, Fig.16, Fig. 17).

Fig.14 Healthy larva of *B. suppressaria*

Fig.15 Healthy larva of *H. talaca*

Fig.16 Bacteria infected dead *B. suppressaria* caterpillar

Fig.17 Bacteria infected dead *H. talaca* caterpillar

B. Mortality of *Buzura suppressaria* due to bacterial infection

The mortality of *B. suppressaria* population collected from tea estates of the Dooars and Terai was observed. Larval samples collected from Terai tea estates (T.E.s) such as Kamalpur, Bengdubi, Atol, Sanyashi, Maruti, Matigara, Nischintapur and

Dagapur, were found to be affected by bacteria at different proportions in different months of the year (Fig.18). Larval populations collected from Nagrakata T.E. and Hantapara T.E. of the Dooars region demonstrated highest mortality rate (8-9%) due to bacterial infection.

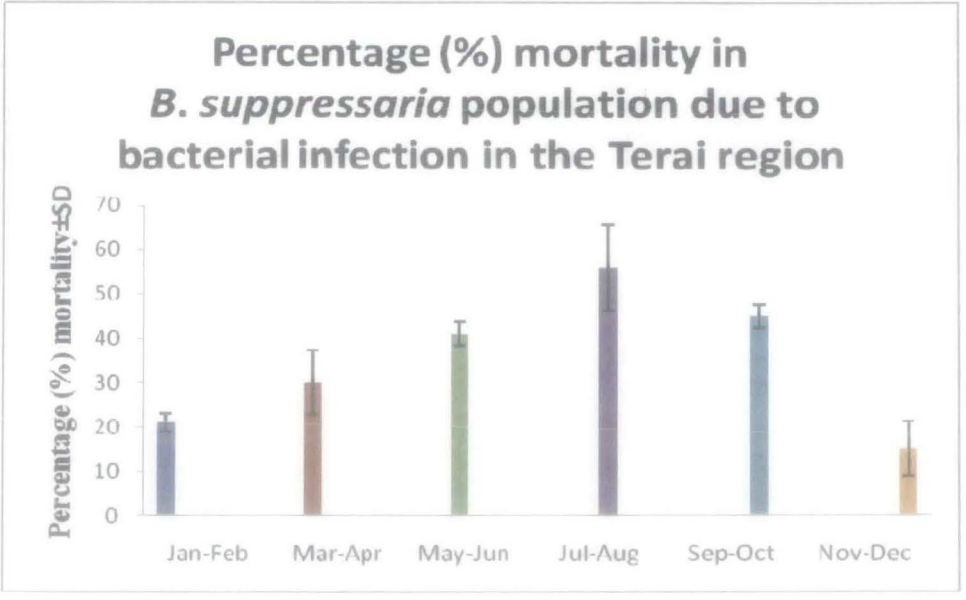


Fig.18 Percent mortality in *B. suppressaria* population due to bacterial infection in the Terai region.

In Chuapara T.E. bacterial infection was not so frequent in population of *B. suppressaria*. In Binnaguri bacterial infection was also recorded. Though the bacterial infection persisted in *B. suppressaria* populations of Terai almost throughout the year, its peak infectivity leading to high mortality was during the rainy months of July-August (Fig.18).

C. Mortality of *Hyposidra talaca* due to bacterial infection

Mortality of *Hyposidra talaca* population due to bacterial infection was mostly recorded in certain T.Es of the Dooars region. The tea estates surveyed for *H. talaca* in the Dooars region were Nagrakata, Hantapara, Chuapara, Bhatkhawa, Mujnai, Binnaguri and Kumargram. Bacterial infection of *H. talaca* population in Hantapara and Nagrakata T.E.s causing highest mortality up to 31% and 40% respectively. Bacterial infection of *H. talaca* population of Chuapara T.E. was very low (6-7%). No bacterial infection was

found in populations of Bhatkhawa and Kumargram T.Es. and in Mujnai T.E. bacterial infection caused a moderate to low mortality. A glimpse of mortality in *H. talaca* population in different tea estates of the Dooars region as observed in spring population of March is represented graphically (Fig.19).

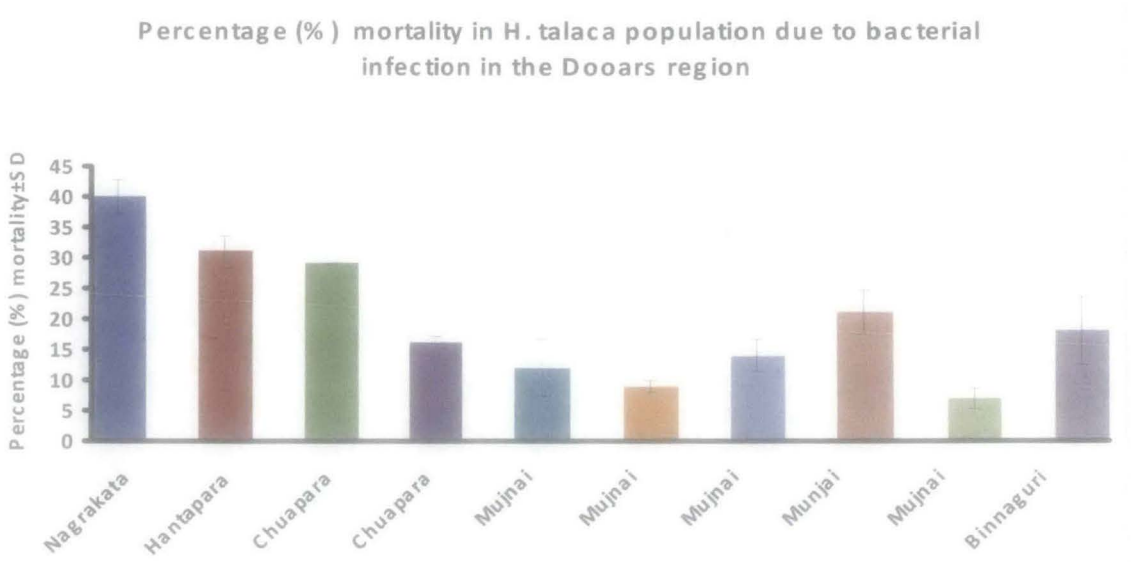


Fig.19 Mortality in natural population of *H. talaca* due to bacterial infection in the Dooars region

D. A glimpse of the Bacterial strains isolated from the loopers (*B. suppressaria* and *H. talaca*)

For the sake of convenience in addressing, describing and discussing the various strains isolated from the two looper species, *B. suppressaria* and *H. talaca* in the following text, these have been given mnemonic designations.

Four isolates from *B. suppressaria* were designated BS01, BS02, BS03, BS04 and ten isolates from *H. talaca* were designated HT01, HT02, HT03, HT04, HT05, HT06, HT07, HT08, HT09 and HT10 (Table.2).

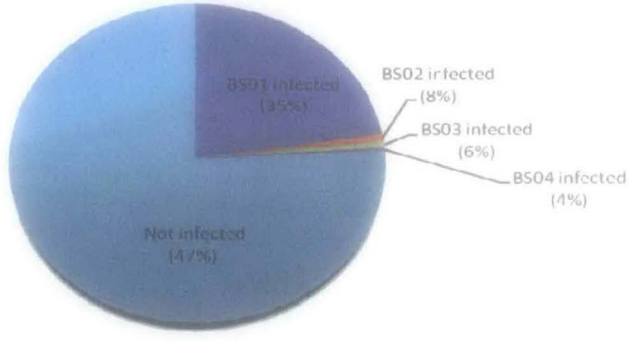
Table.2 Bacterial strains/isolates from loopers at a glance

Name of Tea Pests	Name of bacterial strains isolated
1. *Buzura suppressaria* 2. *Hyposidra talaca*	1. **BS01, BS02, BS03 and BS04** 2. **HT01, HT02, HT03, HT04, HT05, HT06, HT07, HT08, HT09 and HT10**

E. Preliminary characterization and selection of Bacterial strains from *B. suppressaria* and *H. talaca*

Among the fourteen (14) isolated entomopathogenic bacterial strains the most frequently occurring entomopathogens against loopers were BS01 (from *B. suppressaria*), HT01 and HT02 (from *H. talaca*). As such these were selected for detailed study. The rest of the strains occurred occasionally, therefore, only preliminary characterization and Koch's postulate test were performed for them (Fig.14 A, B).

A **Diagramme showing the percentage mortality in *B. suppressaria* population due to different bacterial strains and the percentage of uninfected population**



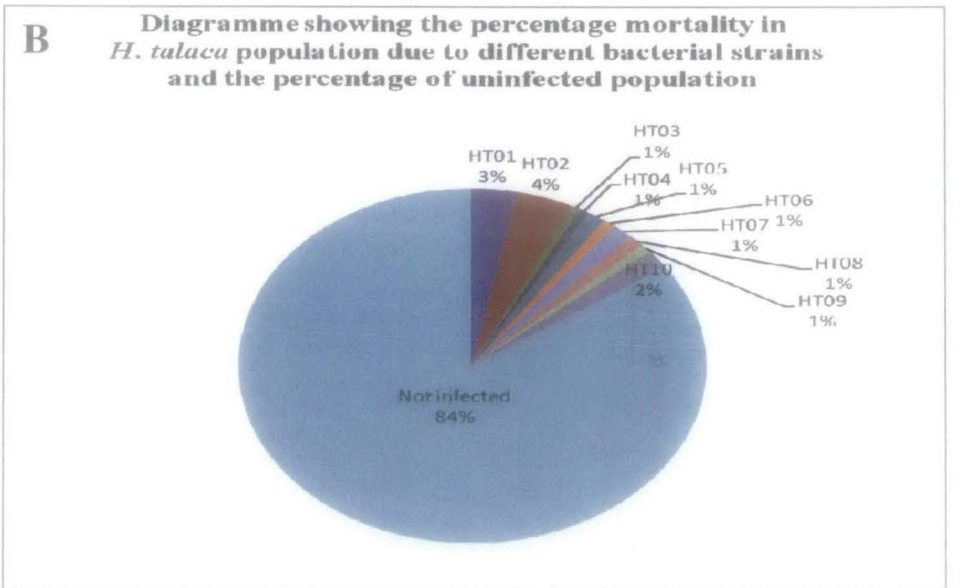


Fig.14 Occurrence of Entomopathogenic bacterial strains in A.) *B. suppressaria* and B.) *H. talaca* populations.

E.a Morphological Characteristics

All the morphological characteristics of a bacterial isolate (BS01) such as vegetative body structure, spore-shape, motility, colony texture, crystal protein shape were found to be similar to *Bacillus thuringiensis kurstaki* (Table.3) (Fig.15 a, b, c, d, e, f and Fig.16). The gram positive facultative anaerobic isolate BS01 showed all the characteristics of genus *Bacillus* (Sneath, 1986) i.e., rod shaped vegetative body, endospore formation, catalase positivity and production of acid from glucose and motility.

Table.3 Comparison of morphological characteristics of BS01 compared with *Btk*.

Morphological Characteristics	*Bacillus thuringiensis kurstaki*	BS01
Vegetative body structur	Rod shaped and Chain like	Rod shaped and Chain like
Motility	Highly motile	Highly motile
Spore shape	Oval	Oval
Crystal protein structure	Bipyramidal	Pyramidal
Colony texture	Smooth	Smooth

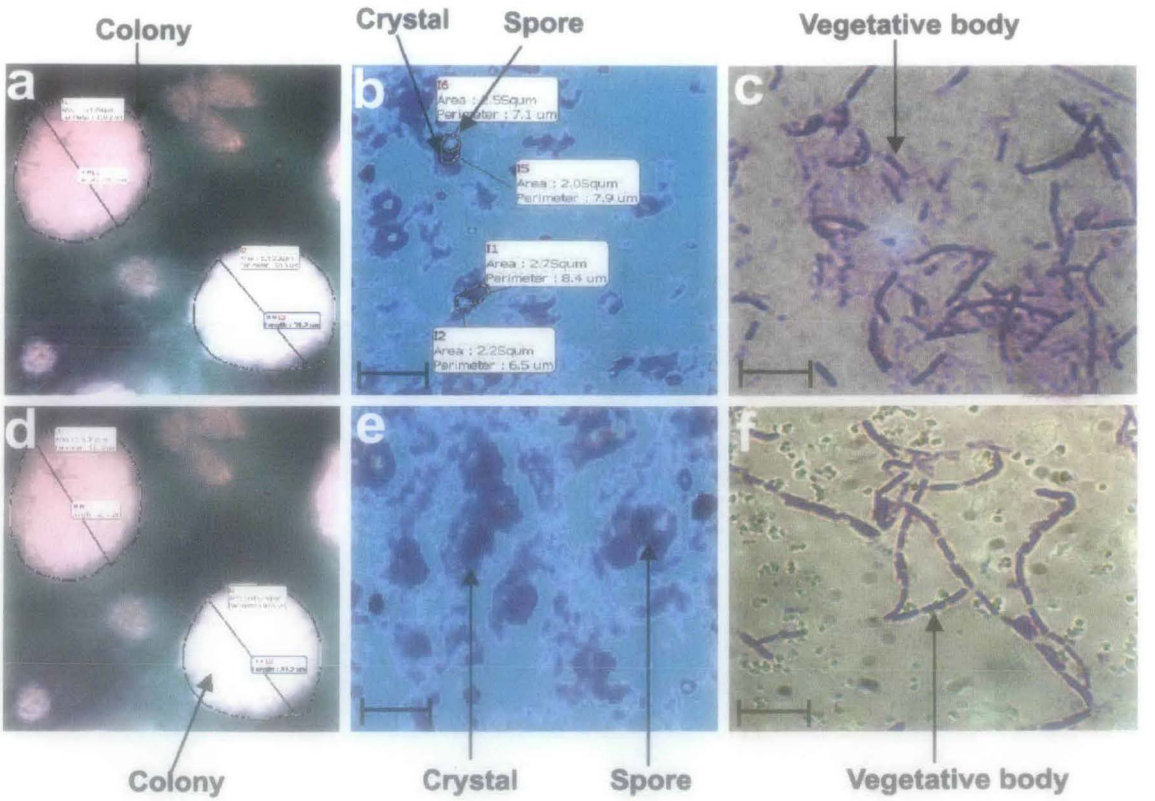


Fig.15 a.) Colonies, b.) Spore and crystal and c.) Vegetative bodies of BS01 isolated from *B. supressaria.* d.) Colonies, e.) Spore and crystal and f.) Vegetative bodies of *Btk* (scale 36µm).

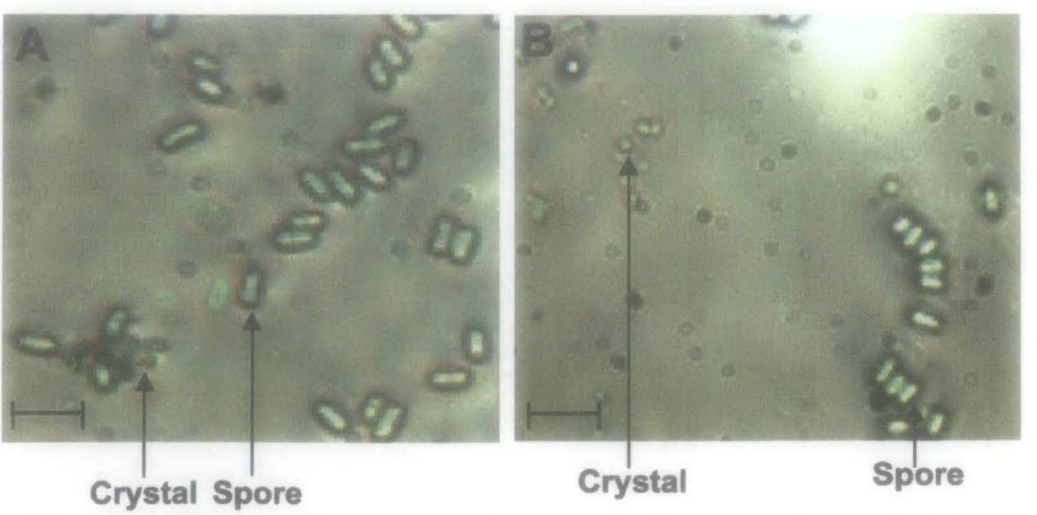


Fig. 16 A.) Phase contrast microphotograph of spore and crystal of BS01 strain and B.) *Btk* (scale 36µm).

The other strains isolated from *B. suppressaria* (BS02, BS03 and BS04) also showed all the characteristics of genus *Bacillus* (Sneath, 1986) i.e., rod shaped vegetative body, gram positivity, endospore formation, catalase positivity and production of acid from glucose and motility.

So, it was found that all the strains isolated from *B. suppressaria* showed the characteristics of genus *Bacillus* as such, these were designated as *Bacillus* sp. BS01, BS02, BS03 and BS04.

E.b Biochemical Characteristics

Biochemical characteristics of BS01 strain showed positive reaction in lysine decarboxylase, ornithin decarboxylase, urease, Voges-Proskaur (V-P) and oxidase tests, and in utilization of trehalose and glucose. The strain showed difference with *Btk* in ONPG, urease, nitrate reduction and oxidase tests. In utilization tests it showed difference in arabinose, xylose, cellobiose, melibiose, saccharose and lactose (Table.4).

Biochemical characteristics of BS02 strain showed positive reaction in nitrate reduction, Voges-Proskaur, esculin hydrolysis and methyl red tests, and in utilization of citrate, arabinose, saccharose, trehalose, glucose and lactose. BS02 showed difference with *Btk* in ONPG, urease, ornithin decarboxylase, lysine decarboxylase and esculin hydrolysis tests. In utilization tests it showed difference in xylose, cellobiose and melibiose. On the other hand BS03 showed positive reaction in ONPG, phenylalanine deamination, V-P, methyl red and esculin hydrolysis tests, and in utilization of citrate, malonate, arabinose, xylose, cellobiose, saccharose, raffinose, trehalose, glucose and lactose. BS03 showed difference with *Btk* in phenyl alanine deamination, nitrate reduction, methyl red and esculin hydrolysis test. In utilization tests it showed difference in citrate, malonate, melibiose and raffinose. BS04 showed positive reaction in ONPG, lysine deacarboxylase, ornithin decarboxylase, urease, nitrate reduction, H_2S production, oxidase and esculin hydrolysis tests, and in utilization of citrate, arabinose, xylose, adonitol, rhamnose, cellobiose, melibiose, raffinose, trehalose, glucose and lactose. It showed difference with *Btk* in urease, oxidase, H_2S, V-P, methyl red and esculin

hydrolysis tests, and in utilization of citrate, malonate, adonitol, rhamnose, saccharose and raffinose (Table.4).

Table.4 Comparative account of biochemical characteristics of *Bacillus* sp. BS01 and *Btk.*

Sl. No.	Name of Biochemical tests	*Btk*	BS01	BS02	BS03	BS04
1.	ONPG	+	-	-	+	+
2.	Lysine decarboxylase	+	+	-	-	+
3.	Ornithin decarboxylase	+	+	-	-	+
4.	Urease	-	+	-	-	+
5.	Phenylalanine deamination	-	-	-	+	-
6.	Nitrate reduction	+	-	+	-	+
7.	H_2S production	-	-	-	-	+
8.	Citrate utilization	-	-	+	+	+
9.	V-P Test	+	+	+	+	-
10.	Methyl red	-	-	+	+	+
11.	Indole	-	-	-	-	-
12.	Malonate	-	-	-	+	+
13.	Esculin hydrolysis	-	-	+	+	+
14.	Arabinose	+	-	+	+	+
15.	Xylose	+	-	-	+	+
16.	Adonitol	-	-	-	-	+
17.	Rhamnose	-	-	-	-	+
18.	Cellobiose	+	-	-	+	+
19.	Melibiose	+	-	-	-	+
20.	Saccharose	+	-	+	+	-
21.	Raffinose	-	-	-	+	+
22.	Trehalose	+	+	+	+	+
23.	Glucose	+	+	+	+	+
24.	Lactose	+	-	+	+	+
25.	Oxidase	-	+	-	-	+

E.c Growth characteristics: determination of generation time

The doubling time was 60 min. in case of BS01 strain of *B. suppressaria*. The doubling time of *Btk* which was used as control reference was much less than the *Bacillus* strain BS01 (Table.5).

Table.5 Comparative account of doubling time of *Btk* and *Bacillus* sp. BS01 strain

Name of Bacteria	Doubling time
***Bacillus* sp. BS01**	**60 mins.**
Btk	**42 mins.**

The doubling time of *Bacillus* sp. BS02 was found to be 72 mins, in case of *Bacillus* sp. BS03 was 54 mins and in case of *Bacillus* sp. BS04 was 51 mins (Table.6).

Table.6 Comparative account of doubling time of *Btk* and *Bacillus* strains (BS02, BS03 and BS04).

Name of Bacteria	Doubling time
***Bacillus* sp. BS02**	**72 mins.**
***Bacillus* sp. BS03**	**54 mins.**
***Bacillus* sp. BS04**	**51 mins.**
Btk	**42 mins.**

E.d SDS-PAGE of crystal protein

When crystal protein of BS01 was analyzed by SDS-PAGE and two major protein bands, 52 and 41 kDa were noticed. Difference was observed for the smaller protein band which was observed as 41 kDa instead of 42 kDa as observed in *Btk* (Fig. 17).

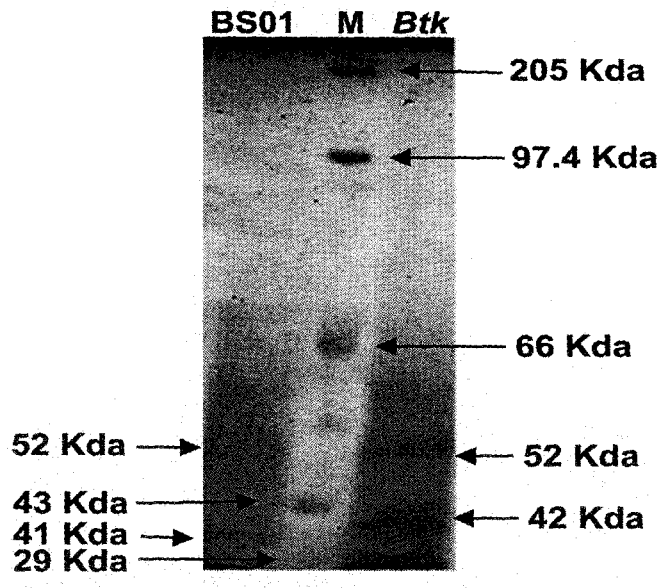


Fig. 17 Difference in banding pattern of BS01 strain of *B. suppressaria* and *Btk* on SDS-PAGE (kDa values indicated by arrows)

E.e Qualitative (SDS-PAGE) analysis of whole body protein profile of *Bacillus* strains

Difference in banding pattern of total protein was observed between *Btk* and BS01 strain. Two major protein bands (44 and 31 kDa) were present in both *Btk* and BS01 strains. Difference was apparent due to the presence of two bands of 33.5 and 34 kDa, in BS01 strain which were absent in *Btk* (Fig. 18).

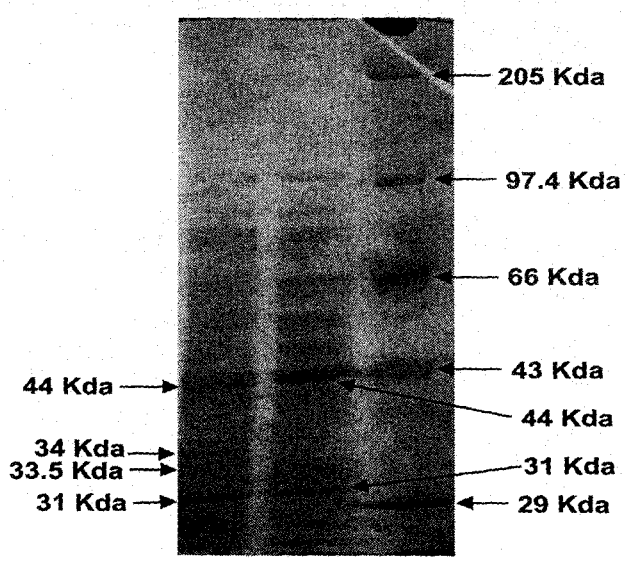


Fig. 18 SDS-PAGE analysis of whole body protein of BS01 strain of *B. suppressaria* and *Btk*

F. Preliminary characterization of bacterial strains from *H. talaca*

F.a Morphological Characteristics

All phenotypic characteristics of bacterial strains (HT01 and HT02) isolated from *H. talaca* like cell and colony morphology, motility, shape of the endospore, crystal protein shape were found to be similar with *Bacillus thuringiensis kurstaki* (Table.7) (Fig.19 a, b, c, d, e, f). The isolates HT01 and HT02 showed all the characteristics of genus *Bacillus* including cell morphology, gram positivity, endospore production, facultative anaerobic, catalase positivity, production of acid from glucose and motility (Sneath, 1986).

Table.7 Comparative account of morphological characteristics of HT01, HT02 with *Btk*.

Morphological Characteristics	*Btk*	HT01 and HT02
Vegetative body structur	Rod shaped and Chain like	Rod shaped and Chain like
Motility	Highly motile	Highly motile
Spore shape	Oval	Oval
Crystal protein structure	Bipyramidal	Oval
Colony texture	Smooth	Smooth

Eight more entomopathogenic bacterial strains (HT03, HT04, HT05, HT06, HT07, HT08, HT09 and HT10) were isolated from *Hyposidra talaca*. These strains occurred occasionally and infrequently in *H. talaca* population. These strains also showed all the characteristics of genus *Bacillus* including rod shaped vegetative body, endospore formation, gram positivity, catalase positivity and production of acid from glucose and motility (Sneath, 1986).

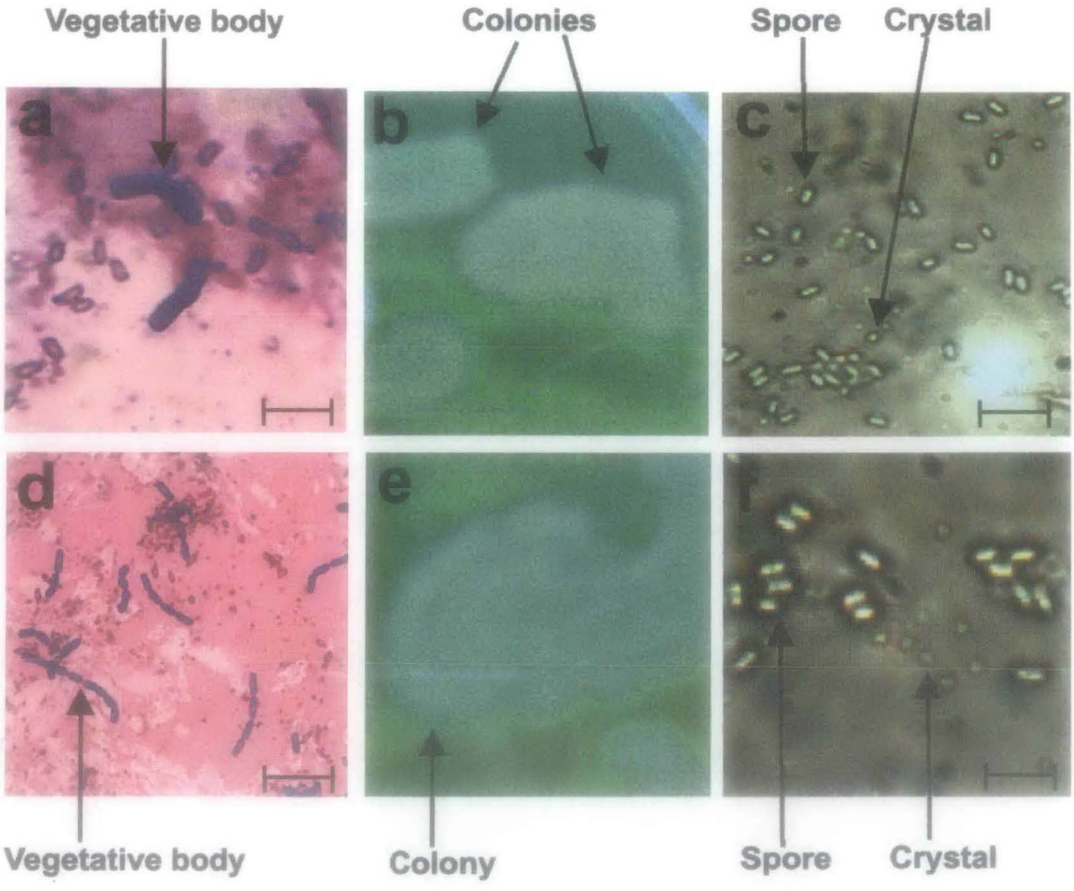


Fig.19 a. Vegetative bodies, b. Colony morphology and c. Phase contrast microscopic photograph of spore and crystal of HT01. d. Vegetative bodies, e. Colony morphology and f. Phase contrast microscopic photograph of spore and crystal of HT02 (scale 36μm).

It was found that all the strains isolated from *H. talaca* showed all the characteristics of genus *Bacillus*, so they were designated as *Bacillus* sp. HT01, HT02, HT03, HT04, HT05, HT06, HT07, HT08, HT09 and HT10.

F.b Biochemical Characteristics

Strain HT01 showed positive reaction in lysine decarboxylase, ornithin decarboxylase, Voges-Proskaur, and urease tests, and in utilization of citrate, malonate, cellobiose, melibiose, saccharose, raffinose, trehalose and glucose. It showed difference with *Btk* in ONPG, urease and nitrate tests and in utilization tests it showed difference in citrate, malonate, arabinose, xylose and lactose. On the other hand HT02 strain showed positive reaction in lysine decarboxylase, ornithin decarboxylase, urease, Voges-Proskaur

and oxidase tests, and in utilization of trehalose and glucose. It showed difference with *Btk* as BS01 strain (Table.8).

Table.8 Comparative account of biochemical characteristics of *Bacillus* sp. HT01, *Bacillus* sp. HT02 and *Btk*

Sl. No.	Name of Biochemical tests	*Btk*	HT01	HT02
1.	ONPG	+	-	-
2.	Lysine decarboxylase	+	+	+
3.	Ornithin decarboxylase	+	+	+
4.	Urease	-	+	+
5.	Phenylalanine deamination	-	-	-
6.	Nitrate reduction	+	-	-
7.	H_2S production	-	-	-
8.	Citrate utilization	-	+	-
9.	V-P Test	+	+	+
10.	Methyl red	-	-	-
11.	Indole	-	-	-
12.	Malonate	-	+	-
13.	Esculin hydrolysis	-	-	-
14.	Arabinose	+	-	-
15.	Xylose	+	-	-
16.	Adonitol	-	-	-
17.	Rhamnose	-	-	-
18.	Cellobiose	+	+	-
19.	Melibiose	+	+	-
20.	Saccharose	+	+	-
21.	Raffinose	-	+	-
22.	Trehalose	+	+	+
23.	Glucose	+	+	+
24.	Lactose	+	-	-
25.	Oxidase	-	-	+

The other eight strains isolated from *H. talaca* showed difference from *Btk* and one another. Strain HT03 showed positive reaction in Voges-Proskaur and esculin hydrolysis tests, and in utilization of citrate, malonate, rhamnose and glucose. It showed

difference with *Btk* in ONPG, lysine, ornithin, nitrate and esculin hydrolysis tests and in utilization tests it showed difference in citrate, malonate, arabinose, xylose, rhamnose, melibiose, cellobiose, trehalose, saccharose and lactose. On the other hand HT04 strain showed positive reaction in ornithin decarboxylase, nitrate, Voges-Proskaur and esculin hydrolysis tests, and in utilization of citrate, malonate and rhamnose. It showed difference with *Btk* in ONPG, lysine decarboxylase and esculin hydrolysis tests, and in utilization of citrate, malonate, arabinose, xylose, rhamnose, cellobiose, melibiose, saccharose, trehalose, glucose and lactose. HT05 showed positive reaction in 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. It showed difference 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. HT06 showed positive reaction in 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. HT07 showed positive reaction in 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 difference with *Btk* in urease, H_2S, V-P, methyl red and esculin hydrolysis tests and in utilization of malonate, adonitol, rhamnose, saccharose and raffinose. HT08 showed positive reaction in 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 difference with *Btk* in urease, V-P and esculin hydrolysis tests and in utilization of citrate, malonate, adonitol, rhamnose and raffinose. HT09 showed positive reaction in nitrate, methyl red and esculin hydrolysis tests and in utilization of citrate, malonate, arabinose, xylose, cellobiose, melibiose, saccharose, trehalose, raffinose and glucose. It showed difference 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. HT10 showed positive reaction in 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 difference 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 (Table.9).

Table.9 Comparative account of biochemical characteristics of eight rare strains of *Bacillus* (HT03 to HT10) isolated from *H. talaca* and *Bacillus thuringiensis kurstaki*.

Sl. No.	Name of Biochemical tests	*Btk*	HT03	HT04	HT05	HT06	HT07	HT08	HT09	HT10
1.	ONPG	+	-	-	-	-	+	+	-	-
2.	Lysine decarboxylase	+	-	-	-	-	+	+	-	-
3.	Ornithin decarboxylase	+	-	+	+	-	+	+	-	+
4.	Urease	-	-	-	+	+	+	+	-	+
5.	Phenylalanine deamination	-	-	-	+	-	-	-	-	-
6.	Nitrate reduction	+	-	+	+	+	+	+	+	+
7.	H_2S production	-	-	-	+	-	+	-	-	-
8.	Citrate utilization	-	+	+	+	-	-	+	+	+
9.	V-P Test	+	+	+	-	-	-	-	-	-
10.	Methyl red	-	-	-	-	+	+	-	+	+
11.	Indole	-	-	-	-	-	-	-	-	-
12.	Malonate	-	+	+	+	+	+	+	-	+
13.	Esculin hydrolysis	-	+	+	+	+	+	+	+	+
14.	Arabinose	+	-	-	+	+	-	+	+	+
15.	Xylose	+	-	-	+	+	+	+	+	+
16.	Adonitol	-	-	-	-	-	+	+	-	-
17.	Rhamnose	-	+	+	-	-	+	+	-	-
18.	Cellobiose	+	-	-	+	+	+	+	+	+
19.	Melibiose	+	-	-	+	+	+	+	+	+
20.	Saccharose	+	-	-	+	+	-	+	+	+
21.	Raffinose	-	-	-	+	+	+	+	+	+
22.	Trehalose	+	-	-	+	+	+	+	+	-
23.	Glucose	+	+	-	+	+	+	+	+	+
24.	Lactose	+	-	-	+	-	+	+	-	-
25.	Oxidase	-	-	-	-	-	-	-	-	-

F.c Growth characteristics: determination of generation time

The doubling time was 48 min. in case of HT01 and HT02 strains. The doubling time of *Btk* which was used as control was totally different from the above two *Bacillus* strains (Table.10).

Table. 10 Comparative account of doubling time of *Bacillus* sp. HT01, HT02 and *Btk*.

Name of Bacterial strains	Doubling time
***Bacillus* sp. HT01**	**48 mins.**
***Bacillus* sp. HT02**	**48 mins.**
Btk	**42 mins.**

It was found that the doubling time for *Bacillus* sp. HT03 was 60 min., for *Bacillus* sp. HT04 was 66 min., for *Bacillus* sp. HT05 was 42 min., for *Bacillus* sp. HT06 was 30 min., for *Bacillus* sp. HT07 was 36 min., for *Bacillus* sp. HT08 was 30 min., for *Bacillus* sp. HT09 was 24 min. and in case of *Bacillus* sp. HT10 was 33 min. (Table.11).

Table. 11 Doubling times of *Bacillus* strains (HT03, HT04, HT05, HT06, HT07, HT08, HT09 and HT10) isolated from *H. talaca*

Name of Bacterial strains	Doubling time
***Bacillus* sp. HT03**	**60 mins.**
***Bacillus* sp. HT04**	**66 mins.**
***Bacillus* sp. HT05**	**42 mins.**
***Bacillus* sp. HT06**	**30 mins.**
***Bacillus* sp. HT07**	**36 mins.**
***Bacillus* sp. HT08**	**30 mins.**
***Bacillus* sp. HT09**	**24 mins.**
***Bacillus* sp. HT10**	**33 mins.**
Btk	**42 mins.**

F.d SDS-PAGE analysis of crystal protein

SDS-PAGE of crystal protein of HT01 strain revealed presence of three distinct protein bands having molecular weights 86, 53 and 40 kDa. Such bands were absent in *Btk*. On the other hand HT02 strain differed from *Btk* in 92, 76, 64, 38 and 30 kDa molecular weight protein bands which were present in HT02 strain but absent in *Btk* (Fig. 20). kDa values for the bands of HT01 were recorded as band no.1: 86 kDa, and no.2: 40 kDa; and for HT02 were recorded as no.3: 92 kDa, no.4: 76 kDa no.5: 64 kDa, no.6: 38 kDa, no.7: 30 kDa (Table. 12).

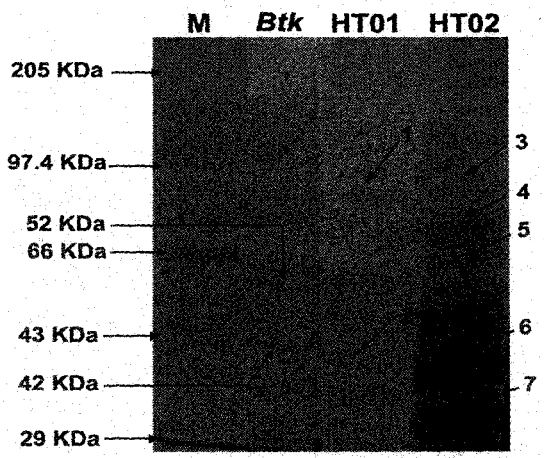


Fig.20 SDS-PAGE profile of crystal protein of *Bacillus* sp. HT01 and *Bacillus* sp. HT02 strains.

Table. 12 Comparison of kDa values of the protein bands of crystal proteins of HT01, HT02 and *Btk* strains.

Values of main crystal protein bands on SDS-PAGE in kDa		
Bacillus sp. HT01	*Bacillus* sp. HT02	*Btk*
86	92	52
40	76	42
-	64	-
-	38	-
-	30	-

F.e Qualitative (SDS-PAGE) analysis of whole body protein of bacterial strains

In case of HT01 and HT02 strains no differences in whole body protein profile were found with *Btk*.

G. Results of bioassay

G.a Bioassay of *Bacillus* strain of *B. suppressaria*

The LC_{50} value for BS01 strain was found to be 446.7µg/ml with fiducial limits 407.96µg/ml (lower limit) and 485.44µg/ml (upper limit). The LT_{50} values for BS01 were found to be 6.19 days for 1000µg/ml, 6.5 days for 750µg/ml and 8.92 days for 500µg/ml concentrations. The LC_{50} value of *Btk*, tested on *B. suppressaria* larvae was found to be 524.8µg/ml with fiducial limits 462.65µg/ml and 586.94µg/ml. The LT_{50} values were found to be 6.35 days for 1000µg/ml and 8.91 days for 750µg/ml concentrations (Table.13).

Table. 13 Results of bioassay of *Bacillus* sp. BS01 strain on *B. suppressaria*

Name of bacteria	%mortality	LC_{50} with Fiducial limits	LT_{50} (days)	Hetero-geneity	Regression
Btk tested on *B.suppressaria*	75% for 1000 µg/ml 60% for 750 µg/ml 48% for 500 µg/ml 18% for 300 µg/ml 17% for 100 µg/ml	524.8µg/ml With 462.65µg/ml (Lower limit) 586.94µg/ml (Upper limit)	6.35 for 1000µg/ml 8.91 for 750µg/ml	$\chi^2(5)$=120 for 1000µg/ml $\chi^2(5)$=85.7143 for 750 µg/ml $\chi^2(5)$=63.1579 for 500 µg/ml $\chi^2(5)$=19.7802 for 300 µg/ml $\chi^2(5)$=18.5792 for100µg/ml	Y=1.669X+2.253
BS01 tested on *B.suppressaria*	81% for 1000µg/ml 59% for 750µg/ml 54 % for 500 µg/ml 6% for 300µg/ml 3% for 100µg/ml	446.7µg/ml With 407.96µg/ml (Lower limit) 485.44µg/ml (Upper limit)	6.19 for 1000µg/ml 6.5 for 750µg/ml 8.92 for 500µg/ml	$\chi^2(5)$=3.0457 for 100µg/ml $\chi^2(5)$=6.1856 for 300µg/ml $\chi^2(5)$=73.9726 for 500µg/ml $\chi^2(5)$=83.6879 for 750µg/ml $\chi^2(5)$=136.1345 for 1000µg/ml	Y=3.33X-21.3

G.b Bioassay of *Bacillus* strains of *H. talaca*

The LC_{50} value for HT01 strain was found to be 166µg/ml with fiducial limits 127.22µg/ml (lower limit) and 204.78µg/ml (upper limit). The LT_{50} values were found to be 4.085 days for 1000µg/ml, 5.65 days for 750µg/ml, 5.68 days for 500µg/ml, 7.90 days for 300µg/ml and 8.4 days for 100µg/ml concentrations. The LC_{50} value for HT02 strain was found to be 239.9µg/ml with fiducial limits 223.37µg/ml (lower limit) and 250.42µg/ml (upper limit). The LT_{50} values were found to be 4.32 days for 1000µg/ml, 5.73 days for 750µg/ml, 8.15 days for 500µg/ml and 8.3 days for 300µg/ml concentrations. The LC_{50} value of *Btk* which was tested on *H. talaca* larvae was found to be 438.19µg/ml with 380.47µg/ml (lower limit) and 504.678µg/ml (upper limit). The LT_{50} values were found to be 5.92 days for 1000µg/ml, 7.52 days for 750µg/ml and 7.68 days for 500µg/ml concentrations. (Table. 14)

Table. 14 Results of bioassay of *Bacillus* sp. HT01 and HT02 strains on *H. talaca*

Name of bacteria	% mortality	LC_{50} with Fiducial limits	LT_{50} (days)	Heterogeneity	Regression
Btk tested on *H. talaca*	76.84% for 1000 µg/ml 71.57% for 750µg/ml 55.78% for 500µg/ml 24.21% for 300µg/ml 14.73% for 100µg/ml	438.19µg/ml With 380.47µg/ml (Lower limit) 504.67µg/ml (Upper limit)	5.92 for 1000µg/ml 7.52 for 750µg/ml 7.68 for 500µg/ml	$\chi^2(5)=109.7518$ for 1000µg/ml $\chi^2(5)=97.1837$ for 750 µg/ml $\chi^2(5)=65.0910$ for 500 µg/ml $\chi^2(5)=19.1980$ for 300 µg/ml $\chi^2(5)=9.2803$ for100µg/ml	Y=5.18X-16.64
HT01 tested on *H. talaca*	84% for 1000µg/ml 80% for 750µg/ml 75% for 500µg/ml 60% for 300µg/ml 52% for 100µg/ml	166µg/ml With 127.22µg/ml (Lower limit) 204.78µg/ml (Upper limit)	4.085 for 1000µg/ml 5.65 for 750µg/ml 5.68 for 500µg/ml 7.90 for 300µg/ml 8.4 for 100µg/ml	$\chi^2(5)=103.8462$ for 1000µg/ml $\chi^2(5)=93.5757$ for 750µg/ml $\chi^2(5)=80.7446$ for 500µg/ml $\chi^2(5)=50.00$ for 300µg/ml $\chi^2(5)=36.7647$ for 100µg/ml	Y=1.079X+2.6
HT02 tested on *H. talaca*	93% for 1000µg/ml 90% for 750µg/ml 73 % for 500µg/ml 54% for 300µg/ml 26% for 100µg/ml	239.9µg/ml With 223.37µg/ml (Lower limit) 250.42µg/ml (Upper limit)	4.32 for 1000µg/ml 5.73 for 750µg/ml 8.15 for 500µg/ml 8.3 for 300µg/ml	$\chi^2(5)=137.9041$ for 1000µg/ml $\chi^2(5)=128.00$ for 750µg/ml $\chi^2(5)=81.7424$ for 500µg/ml $\chi^2(5)=44.4853$ for 300µg/ml $\chi^2(5)=8.6721$ for 100µg/ml	Y=2.554X-1.123

H. Results of cross-infectivity of bacterial strains to other lepidopteran tea pests

H.a Cross infectivity of *Bacillus* strain BS01 to *H. talaca* and *C. theivora*

H.a.i To *H. talaca* caterpillar

The percent mortality of second instar *H. talaca* caterpillars varied between 2 and 73% within 9 days of treatment. The LC_{50} value was found to be 741.3µg/ml with fiducial limits 705.91µg/ml and 776.69µg/ml.

The LT_{50} values were found to be 6.5 days for 1000µg/ml and 6.92 days for 750 µg/ml concentrations (Table. 15).

H.a.ii To *C. theivora* caterpillar

The percent mortality of second instar *C. theivora* caterpillars varied between 31 and 66% within 9 days of treatment. The LC_{50} value was found to be 398.1µg/ml with fiducial limits 396.758µg/ml and 399.442µg/ml.

The LT_{50} values were found to be 7.85 days for 1000µg/ml, 7.92 days for 750 µg/ml and 8.12 days for 500µg/ml concentrations (Table. 16).

Table. 15 Results of cross infectivity of *Bacillus* sp. BS01 to *H. talaca*

Concentration of crude spore crystal mixture (µg/ml) of *Bacillus* sp. BS01	No. of tested larvae (2^{nd} instar) (n)	Actual mortality	Percentage mortality (%)	LT_{50} (days)
1000	100	73	73%	6.5
750	100	52	52%	6.92
500	100	20	20%	-
300	100	10	10%	-
100	100	2	2%	-
CONTROL	100	00	00	-

Heterogeneity	Regression	LC_{50}	Fiducial limits
$\chi 2(5)$= 2.0202 for 100µg/ml $\chi 2(5)$=10.5263 for 300 µg/ml $\chi 2(5)$= 22.2222 for 500 µg/ml $\chi 2(5)$=70.2703 for 750 µg/ml $\chi 2(5)$=114.9606 for 1000 µg/ml	Y=2.601X-12.4	741.3 µg/ml	705.91 µg/ml (Lower limit) 776.69 µg/ml (Upper limit)

Table. 16 Result of cross infectivity of *Bacillus* sp. BS01 to *C. theivora*

Concentration of crude spore crystal mixture (µg/ml)of *Bacillus* sp. BS01	No. of tested larvae (2^{nd} instar) (n)	Actual mortality	Percentage mortality (%)	LT_{50} (days)
1000	100	66	66%	7.85
750	100	58	58%	7.92
500	100	51	51%	8.12
300	100	47	47%	-
100	100	31	31%	-
CONTROL	100	00	00	-

Heterogeneity	Regression	LC_{50}	Fiducial limits
$\chi 2(5)=36.6864$ for 100µg/ml $\chi 2(5)=61.4379$ for 300 µg/ml $\chi 2(5)= 68.4564$ for 500 µg/ml $\chi 2(5)= 81.6901$ for 750 µg/ml $\chi 2(5)=98.5075$ for 1000 µg/ml	Y=47X-587.35	398.1 µg/ml	396.758 µg/ml (Lower limit) 399.442 µg/ml (Upper limit)

H.b Cross infectivity of *Bacillus* strain HT01 to *B. Suppressaria* and *C. theivora*

H.b.i To *B. suppressaria* caterpillar

The percent mortality of second instar *B. suppressaria* caterpillars varied between 22 and 86% within 9 days. The LC_{50} value was found to be 288.4µg/ml with fiducial limits 257.3µg/ml and 319.5µg/ml.

The LT_{50} values were found to be 5.63 days for 1000µg/ml, 7.45 days for 750 µg/ml, 7.57 days for 500µg/ml and 8.77 days for 300µg/ml (Table. 17).

H.b.ii To *C. theivora* caterpillar

The percent mortality of second instar *C. theivora* caterpillars varied between 10 and 72% within 9 days. The LC_{50} value was found to be 457.1µg/ml with fiducial limits 420.86µg/ml and 493.34µg/ml.

The LT_{50} values were found to be 5.92 days for 1000µg/ml, 7.69 days for 750µg/ml and 8.31 days for 500µg/ml concentrations (Table. 18).

Table. 17 Result of cross infectivity of *Bacillus* sp. HT01 to *B. suppressaria*

Concentration of crude spore crystal mixture (µg/ml)of *Bacillus* sp. HT01	No. of tested larvae (2^{nd} instar) (n)	Actual mortality	Percentage mortality (%)	LT_{50} (days)
1000	100	86	86%	5.63
750	100	74	74%	7.45
500	100	70	70%	7.57
300	100	62	62%	8.77
100	100	22	22%	-
CONTROL	100	10	10%	-

Heterogeneity	Regression	LC_{50}	Fiducial limits
$\chi 2(5)=5.3571$ for 100µg/ml $\chi 2(5)=58.6806$ for 300 µg/ml $\chi 2(5)= 75.0000$ for 500 µg/ml $\chi 2(5)= 84.0722$ for 750 µg/ml $\chi 2(5)=115.7051$ for 1000 µg/ml	Y=1.769X+3.21	288.4 µg/ml	257.3 µg/ml (Lower limit) 319.5 µg/ml (Upper limit)

Table. 18 Result of cross infectivity of *Bacillus* sp. HT01 to *C. theivora*

Concentration of crude spore crystal mixture (µg/ml)of *Bacillus* sp. HT01	No. of tested larvae (2^{nd} instar) (n)	Actual mortality	Percentage mortality (%)	LT_{50} (days)
1000	100	72	72%	5.92
750	100	69	69%	7.69
500	100	55	55%	8.31
300	100	30	30%	-
100	100	10	10%	-
CONTROL	100	00	00	

Heterogeneity	Regression	LC_{50}	Fiducial limits
$\chi 2(5)$= 10.5263 for 100µg/ml $\chi 2(5)$= 35.2941 for 300 µg/ml $\chi 2(5)$=75.8621 for 500 µg/ml $\chi 2(5)$= 105.3435 for 750 µg/ml $\chi 2(5)$= 112.5000 for 1000 µg/ml	Y=1.950X-0.998	457.1 µg/ml	420.86 µg/ml (Lower limit) 493.34 µg/ml (Upper limit)

H.c Cross infectivity of *Bacillus* strain HT02 to *B. suppressaria* and *C. theivora*

H.c.i To *B. suppressaria* caterpillar

The percent mortality of second instar *B. suppressaria* caterpillars varied between 30 and 80% within 9 days. The LC_{50} value was found to be 354.8µg/ml with fiducial limits 314.9µg/ml and 394.7µg/ml.

The LT_{50} values were found to be 5.64 days for 1000µg/ml, 7.76 days for 750 µg/ml and 8 days for 500µg/ml concentrations (Table. 19).

H.c.ii To *C. theivora* caterpillar

The percent mortality of second instar *C. theivora* caterpillars varied between 2 and 60% within 9 days. The LC_{50} value was found to be 594.3µg/ml with fiducial limits 589.68µg/ml and 598.92µg/ml.

The LT_{50} values were found to be 6 days for 1000µg/ml and 7.04 days for 750µg/ml concentrations (Table. 20).

Table. 19 Result of cross infectivity of *Bacillus* sp. HT02 to *B. suppressaria*

Concentration of crude spore crystal mixture (µg/ml) of *Bacillus* sp. HT02	No. of tested larvae (2^{nd} instar) (n)	Actual mortality	Percentage mortality (%)	LT_{50} (days)
1000	100	80	80%	5.64
750	100	77	77%	7.76
500	100	68	68%	8
300	100	50	50%	-
100	100	30	30%	-
CONTROL	100	15	15%	-

Heterogeneity	Regression	LC_{50}	Fiducial limits
$\chi 2(5)=27.9209$ for 300 µg/ml $\chi 2(5)= 57.8519$ for 500 µg/ml $\chi 2(5)= 77.3752$ for 750 µg/ml $\chi 2(5)= 84.7118$ for 1000 µg/ml	Y=1.663X+3.742	354.8 µg/ml	314.9 µg/ml (Lower limit) 394.7 µg/ml (Upper limit)

Table. 20 Result of cross infectivity of *Bacillus* sp. HT02 to *C. theivora*

Concentration of crude spore crystal mixture (µg/ml)of *Bacillus* sp. HT02	No. of tested larvae (2^{nd} instar) (n)	Actual mortality	Percentage mortality (%)	LT_{50} (days)
1000	100	60	60%	6
750	100	57	57%	7.04
500	100	30	30%	-
300	100	5	5%	-
100	100	2	2%	-
CONTROL	100	00	00	-

Heterogeneity	Regression	LC_{50}	Fiducial limits
$\chi 2(5)$=2.0202 for 100 µg/ml $\chi 2(5)$=5.1282 for 300 µg/ml $\chi 2(5)$=35.2941 for 500 µg/ml $\chi 2(5)$=79.7203 for 750 µg/ml $\chi 2(5)$=85.7143 for 1000 µg/ml	Y=2.558X-12.11	594.3 µg/ml	589.68 µg/ml (Lower limit) 598.92 µg/ml (Upper limit)

I. Cross infectivity to beneficial lepidopteran (silk worm)

I.a Cross infectivity of *Bacillus* strains of *B. suppressaria* and *H. talaca* to silk worm

In this experiment no notable mortality due to treatment with lower as well as in higher concentrations of the *Bacillus* strains isolated from *B. suppressaria* and *H. talaca* could be recorded.

J. Field trials on biocontrol efficacy

Among the bacteria isolated from the dead and diseased looper caterpillars, the one isolated from *Hyposidra talaca* i.e. HT01 was a highly pathogenic strain. Its LC_{50} and LT_{50} values were found to be lower than the other isolated strains from *B. suppressaria* and *H. talaca.* So a field level experiment was carried out to know the efficacy of the *Bacillus* strain HT01 in the field condition. The study was carried out in a tea estate of the Terai region where pesticide application was temporarily suspended. The experiment was conducted in the month of March. Four treatments (5000µg/ml, 4000µg/ml, 3000µg/ml and 2000µg/ml) with three replications for each concentration were executed along with water spray as control. The count of residual live larvae on seventh day after spraying revealed that the highest concentration (5000µg/ml) was significantly effective compared to other concentrations. The mean percentage live larvae recovered after treatment was recorded on 3^{rd}, 5^{th} and 7^{th} days and a graph was plotted (Mean±SD) (Fig. 21 A, B, C). Analysis based on one-way ANOVA, revealed that the mean percentage of live larvae was recovered from each treatment after 3^{rd}, 5^{th} and 7^{th} day were significantly different from each other and also from control (Table. 21 A, B, C). The lowest percentage of live larvae recovered from the highest concentration sprayed. Control plots had significantly more percentage of live larvae than the treated plots. The treatment with 5000µg/ml showed the best control action. This result showed that the concentrations of bacteria in water formulation applied is crucial for effectiveness as biopesticide against *Hyposidra talaca* larvae without any additives (stickers and spreaders).

Table. 21 A. Comparison of varying doses of *Bacillus* sp. HT01 strain on survival of looper larvae on 7th day.

Treatments (µg/ml)	No. of relicate	Percent live larvae recovered on 7th day after treatment	Mean % of live larvae recovered after 7 days (Mean±SD)
5000	1	3.03 (0.17496)	3.93±1.40
	2	5.55 (0.23782)	
	3	3.22 (0.180421)	
4000	1	6.59 (0.259617)	7.48±3.84
	2	11.7 (0.3491)	
	3	4.16 (0.205402)	
3000	1	11.95 (0.352972)	14±1.92
	2	15.78 (0.408508)	
	3	14.28 (0.357315)	
2000	1	30.92 (0.589635)	32.21±5.70
	2	38.46 (0.668948)	
	3	27.27 (0.549427)	
Control	1	84.04 (1.159825)	91.13±6.20
	2	95.6 (1.359465)	
	3	93.75 (1.318116)	

F = 129.82859

p = 1.42132E-8

The means are significantly different at 0.05 level.

Data in the parentheses were arcsine transformed values taken for ANOVA study.

21 A

Table. 21 B. Comparison of varying doses of *Bacillus* sp. HT01 strain on survival of looper larvae on 5th day.

Treatments (µg/ml)	No. of relicate	Percent live larvae recovered on 5th day after treatment	Mean % of live larvae recovered after 5 days (Mean±SD)
5000	1	65.65 (0.944573)	71.36±7.27
	2	68.88 (0.979)	
	3	79.56 (1.101671)	
4000	1	98.9 (1.465722)	95.06±3.37
	2	92.55(1.294341)	
	3	93.75(1.318116)	
3000	1	98.91(1.466203)	97.91±0.99
	2	97.89(1.425022)	
	3	96.93 (1.394673)	
2000	1	92.78 (1.298753)	92.55±4.52
	2	87.91 (1.215672)	
	3	96.96 (1.395545)	
Control	1	95.74 (1.362904)	97.86±1.83
	2	98.9 (1.465722)	
	3	98.95 (1.468147)	

F = 16.13757

p = 2.31432E-4

The means are significantly different at 0.05 level.

Data in the parentheses were arcsine transformed values taken for ANOVA study.

21 B

Table. 21 C. Comparison of varying doses of *Bacillus* sp. HT01 strain on survival of looper larvae on 3rd day.

Treatments (µg/ml)	No. of relicate	Percent live larvae recovered on 3rd day after treatment	Mean % of live larvae recovered after 3 days (Mean±SD)
5000	1	92.92 (1.301469)	95.82±2.56
	2	97.77 (1.420904)	
	3	96.77 (1.390092)	
4000	1	98.9 (1.465722)	97.17±1.60
	2	95.74(1.362904)	
	3	96.87 (1.392942)	
3000	1	100 (1.465722)	99.65±0.59
	2	100(1.570796)	
	3	98.97 (1.469132)	
2000	1	98.96(1.468638)	99.28±0.61
	2	98.9(1.465722)	
	3	100 (1.570796)	
Control	1	100 (1.570796)	99.28±0.62
	2	98.9 (1.465722)	
	3	98.95 (1.468147)	

F = 4.35705

p = 0.02691

At the 0.05 level, the means are significantly different.

Data in the parentheses were arcsine transformed values taken for ANOVA study.

21 C

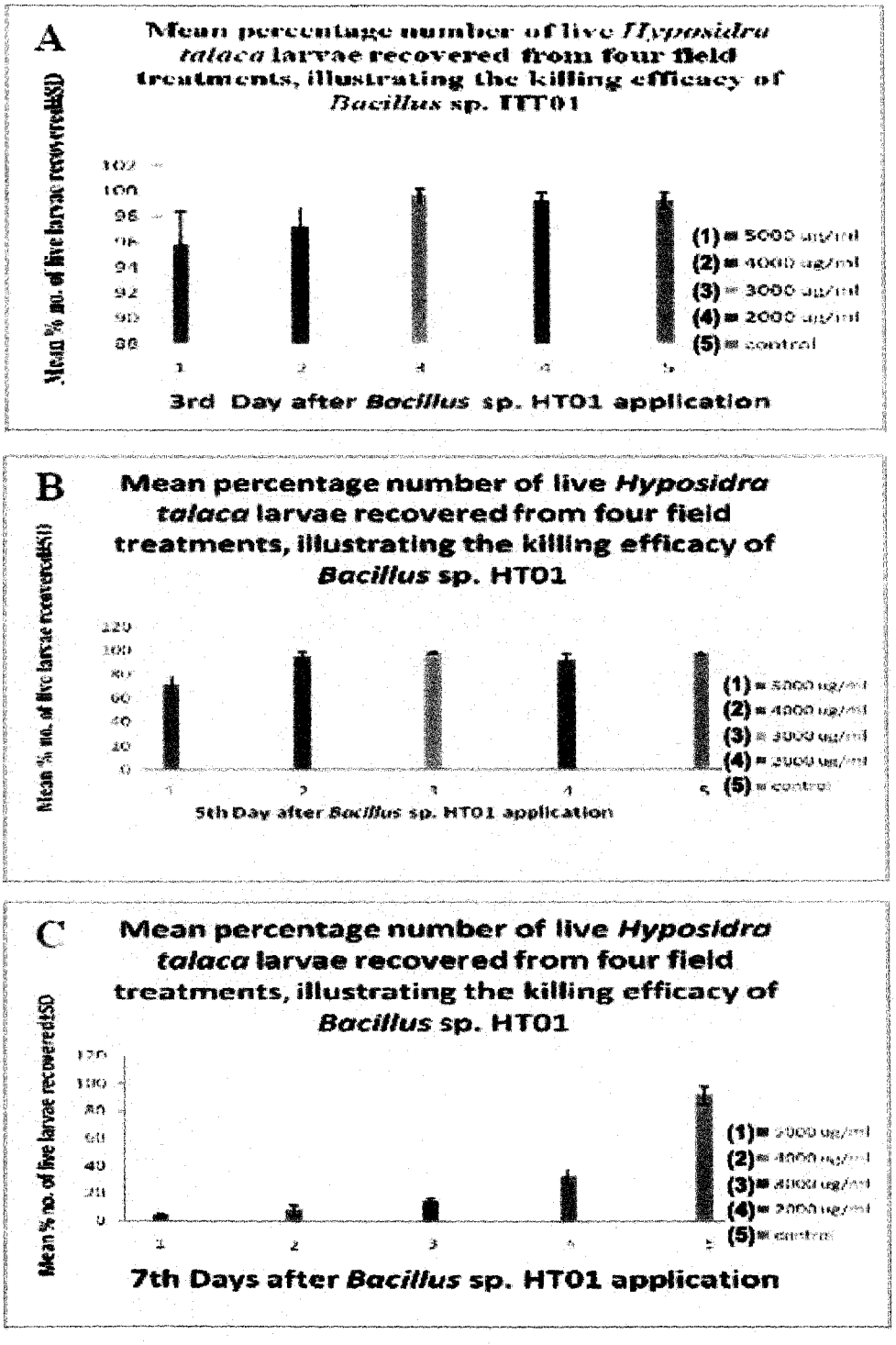


Fig. 21 A., B. and C. Graphs showing the Mean percentage of live larvae recovered on 3rd, 5th and 7th day after treatment

(II) Caloptilia theivora

A. Symptom of bacteria infected larvae

Marked difference could be noted between a healthy larva and a bacteria infected dead larva. The bacteria infected dead larvae turned characteristically blackish in colour with significant shrinkage of body followed by rapid decomposition (Fig. 22, Fig. 23).

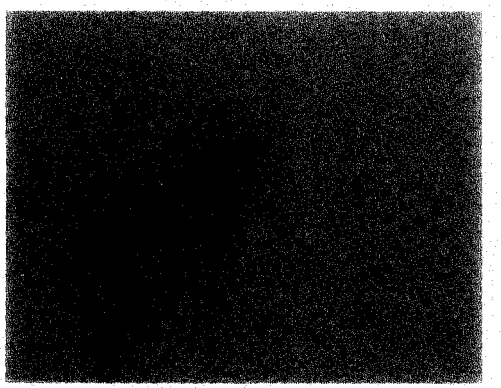


Fig. 22 Healthy *C. theivora* larva

Fig. 23 Bacteria infected dead *C. theivora* larva

B. Mortality of *Caloptilia theivora* due to bacterial infection

The mortality rate due to bacterial infection of *C. theivora* populations collected from different tea estates of Terai and the Dooars were studied. Mortality of the said leaf rollers, collected in various months of the year was noted after bringing to laboratory.

Leaf roller (*Caloptilia theivora*) was an occasional tea pest. The percentage mortality in *Caloptilia theivora* population was more during wetter months. A graphical representation of the percentage mortality due to bacterial infection in the Terai region in different months of a year is represented graphically (Fig. 24)

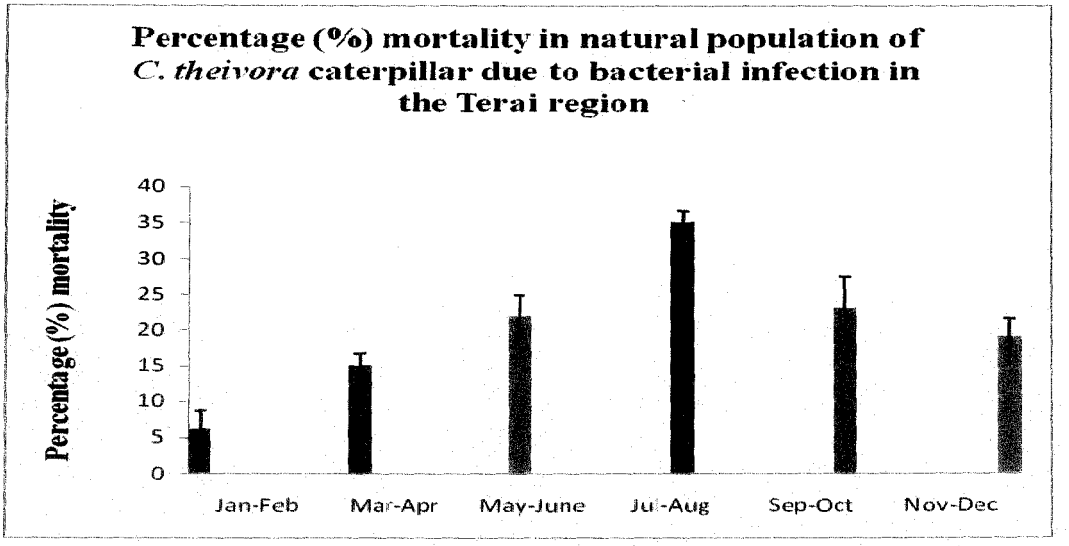


Fig. 24 Percent mortality of *C. theivora* caterpillar due to bacterial infection in the Terai region.

From the histogram (Fig. 24) it was clear that lowest percentage mortality could be recorded in the month of January –February (6%). The highest percentage mortality was in the month of July-August (35%) that is during the rainy season of North Bengal.

Mortality of *C. theivora* populations, collected from two tea estates of the Dooars were 25% (Hantapara T.E.) and 22.85% (Bhatkhawa T.E.).

C. A glimpse of the Bacterial strains isolated from the leaf roller (*Caloptilia theivora*)

For the sake of convenience in addressing, describing and discussing the various strains isolated from the leaf roller species, *Caloptilia theivora* in the following text, these have been given mnemonic designations.

Four strains/isolates from *C. theivora* were designated CT01, CT02, CT03, CT04 and DD01 (Table. 22).

Table. 22 Entomopathogenic bacteria isolated from cadaver of *C. theivora* at a glance.

Name of Tea Pest	Name of Bacteria isolated
Caloptilia theivora	CT01, CT02, CT03, CT04 and DD01

D. Preliminary characterization of entomopathogenic bacterial strains

D.a *Bacillus* strains

D.a.i Morphological Characteristics

All phenotypic characteristics like cell and colony morphology, motility, shape of the endospore of the isolated bacterial strains (CT01, CT02, CT03 and CT04), were found to be similar to *Bacillus thuringiensis kurstaki* except crystal protein shape (Table. 23, Fig. 25 A, B, C, D, E, F, G; Fig. 26 A, B, C; Fig. 27 A, B, C and Fig. 28 A, B, C). The characteristics of genus *Bacillus* (Sneath, 1986) i.e. cell morphology, gram positivity, endospore formation, facultative anaerobic, catalase positivity, acid production from glucose and motility.

So, it was found that all the strains isolated from *C. theivora* showed the characteristics of genus *Bacillus* as such these were designated as *Bacillus* sp. CT01, CT02, CT03 and CT04.

Table. 23 Comparision of morphological characteristics of *Bacillus* sp. CT01, CT02, CT03 and CT04 with *Btk*.

Morphological Characteristics	*Btk*	*Bacillus* sp. CT01, CT02, CT03 and CT04
Vegetative body Structure	Rod shaped and Chain like	Rod shaped and Chain like
Motility	Highly motile	Highly motile
Spore shape	Oval	Oval
Crystal protein Structure	Bipyramidal	Pyramidal for CT01, oval for CT02, bipyramidal for CT03 and CT04 strains
Colony texture	Smooth	Smooth

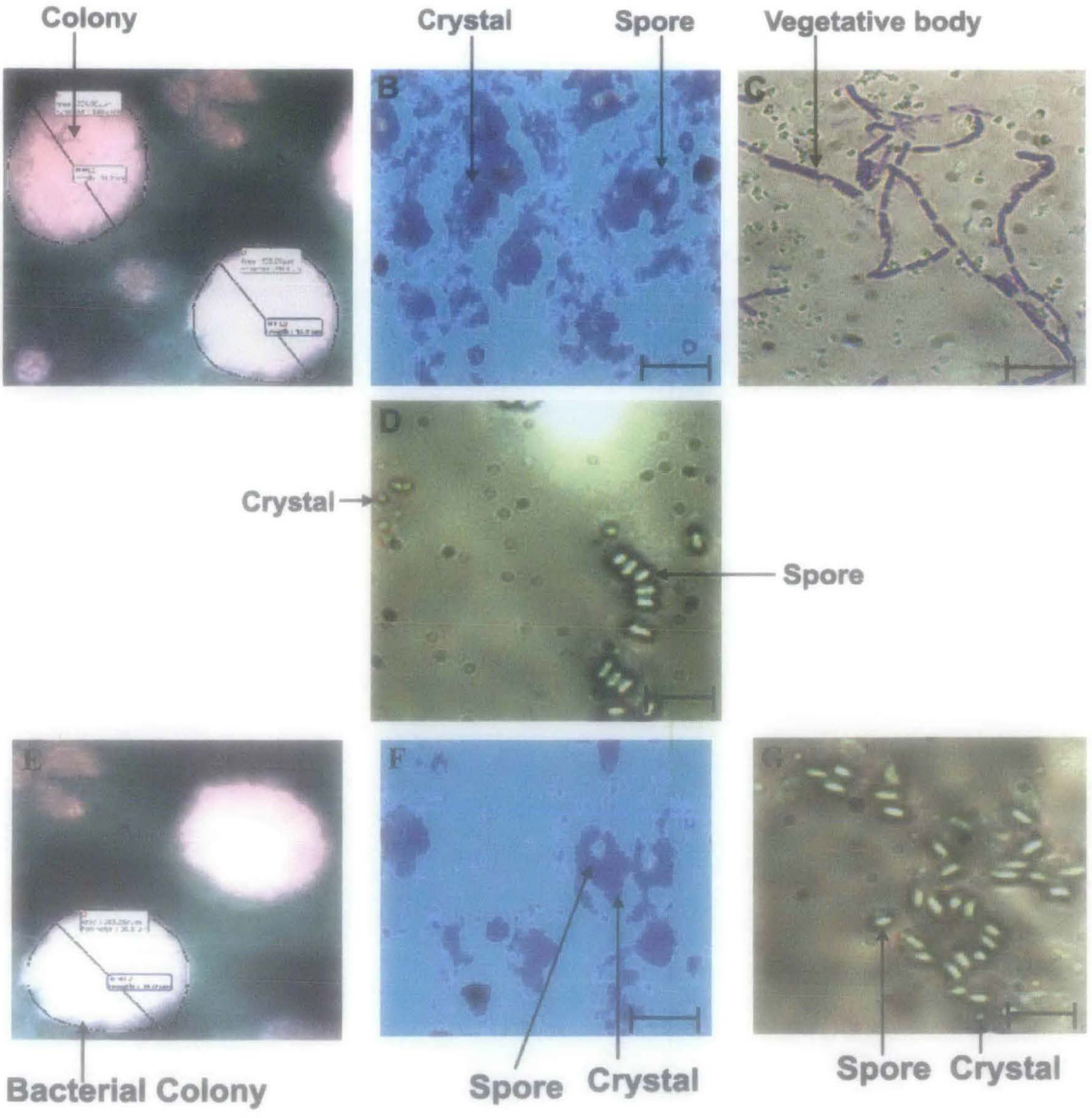


Fig. 25 A. Colony morphology, B. Spore and crystal, C. Vegetative body and D. Phase contrast microphotograph of spores and crystal of *Btk*, E. Colony morphology, F. Spore and crystal and G. Phase contrast microphotograph of spores and crystal of *Bacillus* sp. CT01 isolated from *C. theivora* (scale 36µm).

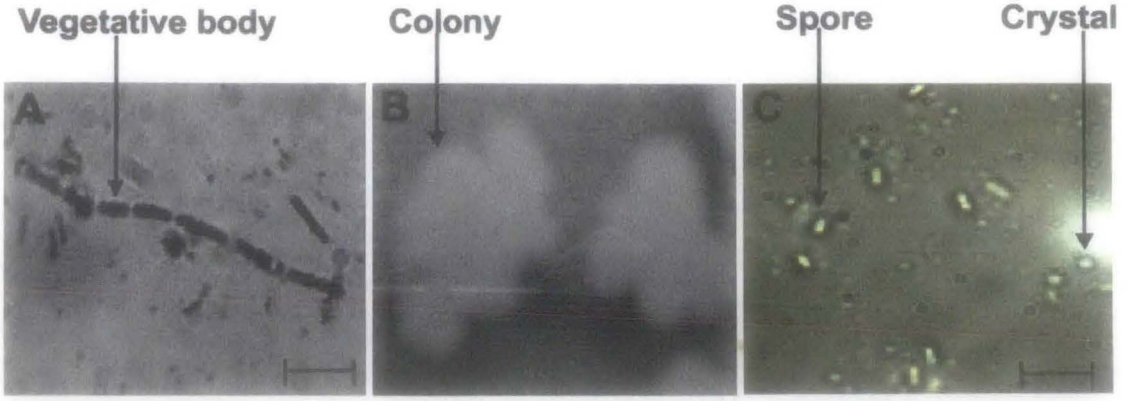


Fig. 26 A. Vegetative body, B. Colony morphology and C. Spore and Crystal (Phase Microscopic view) of *Bacillus* sp. CT02 isolated from *C. theivora*(scale 36µm)

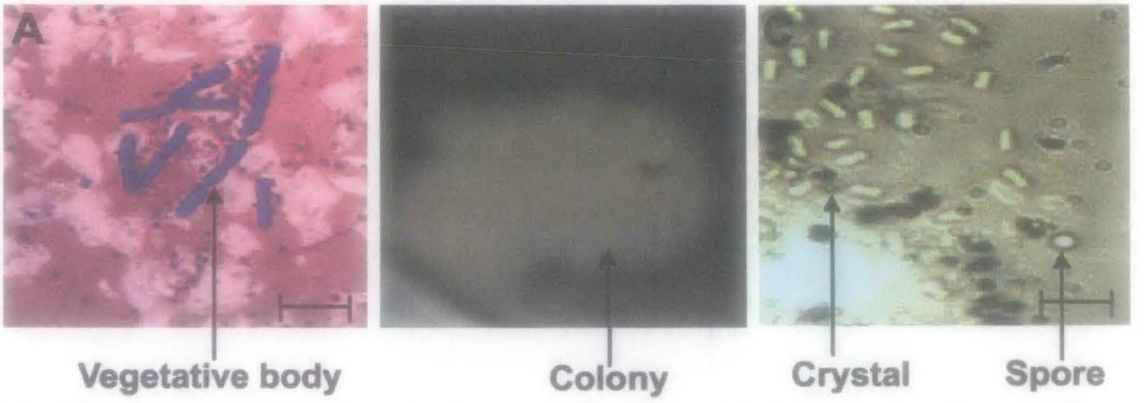


Fig. 27 A. Vegetative body, B. Colony morphology and C. Spore and Crystal (Phase contrast microscopy) of *Bacillus* sp. CT03 isolated from *C. theivora*(scale 36µm)

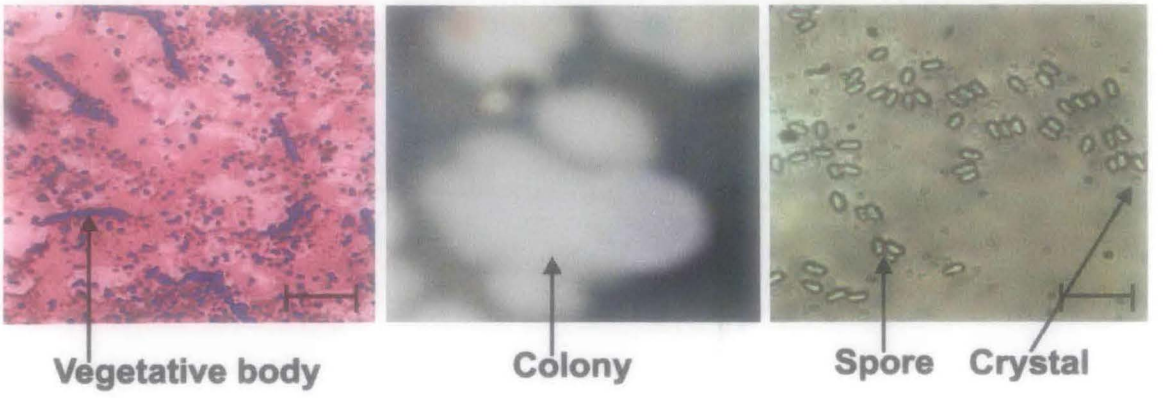


Fig. 28 A. Vegetative body, B. Colony morphology and C. Spore and Crystal (Phase contrast microscopy) of *Bacillus* sp. CT04 isolated from *C. theivora* (scale 36µm)

D.a.ii Biochemical Characteristics

In biochemical characteristics, CT01 strain showed positive reaction in lysine decarboxylase, ornithin decarboxylase, urease, Voges-Proskaur and oxidase tests, and in utilization of trehalose and glucose. However, CT01 differed from *Btk* in ONPG, urease, nitrate and oxidase tests. Further, it showed difference in utilization tests of arabinose, xylose, cellobiose, melibiose, saccharose and lactose. Strain CT02 showed positive reaction in lysine decarboxylase, ornithin decarboxylase, nitrate reduction, Voges-Proskaur, and urease tests, and in utilization of citrate, saccharose, trehalose and glucose. It showed difference with *Btk* in ONPG, and urease tests, and in utilization tests of citrate, arabinose, xylose, cellobiose, melibiose and lactose. On the other hand, CT03 strain showed positive reaction in 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 difference with *Btk* in urease and esculin hydrolysis tests, and in utilization tests it showed difference in citrate, malonate, arabinose, raffinose and lactose. In case of CT04, the strain showed positive reaction in 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 difference with *Btk* in urease and esculin hydrolysis tests and in utilization tests it showed difference in citrate, malonate, cellobiose, raffinose, glucose and lactose (Table. 24).

Table. 24 Comparison of biochemical characteristics of CT01, CT02, CT03, CT04 and *Btk*.

Biochemical tests	*Btk*	CT01	CT02	CT03	CT04
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	-	+	-	-	-

D.a.iii Growth characteristics: determination of generation time

The doubling time was 132 min in case of CT01, 78 min in case of CT02, 42 min in case of CT03, 66 min in case of CT04 and 42 min in case of *Btk*. (Table. 25).

Table. 25 Comparison of growth characteristics of CT01, CT02, CT03, CT04 strains with *Btk*.

Name of Bacterial strains isolated	Doubling time
Bacillus sp. CT01	132 mins.
Bacillus sp. CT02	78 mins.
Bacillus sp. CT03	42 mins.
Bacillus sp. CT04	66 mins.
Btk	42 mins.

D.a.iv SDS-PAGE profile of crystal protein of bacteria

When crystal protein composition was analyzed by SDS-PAGE, CT01 showed one major protein band having the molecular weight 51 kDa. This protein band was absent in *Btk* where as 52 kDa protein band was present in *Btk*, which was again absent in CT01 strain. So, a difference in banding pattern was found between CT01 and *Btk*. In CT02 strain two protein bands having molecular weight 37 kDa and 31 kDa were found. In CT03 a major protein band 118 kDa was found which was absent in all the other three strains and *Btk*. In CT04 strain 38 kDa and 29 kDa protein bands were found (Fig. 29).

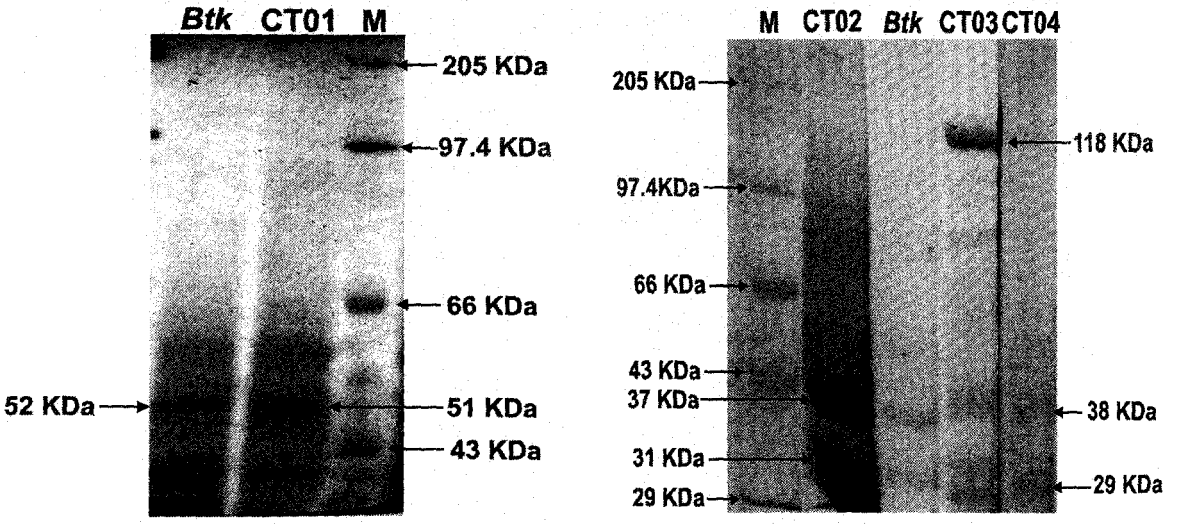


Fig. 29 SDS-PAGE analysis of crystal protein of four *Bacillus* strains isolated from *C. theivora* with *Btk*.

D.a.v Qualitative (SDS-PAGE) analysis of whole body protein of bacteria

No differences were found in whole cell protein profile of CT01, CT03 and CT04 strains with *Btk* in SDS- PAGE. Only an additional protein band having molecular weight 34 kDa was found in CT02 strain (Fig. 30).

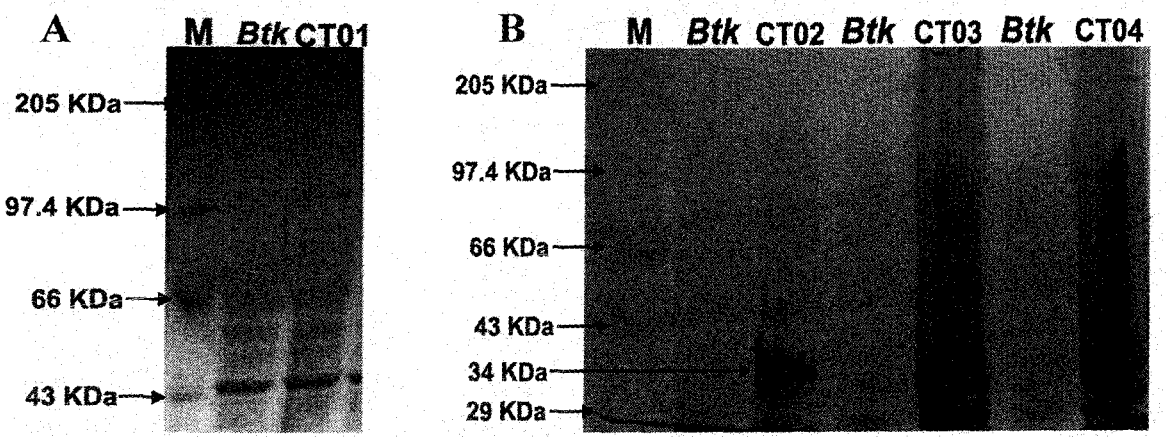


Fig. 30 SDS-PAGE analysis of vegetative protein of A. *Bacillus* sp. CT01, B. *Bacillus* sp. CT02, CT03 and CT04 compared with *Btk*

D.b *Enterobacter* strain

D.b.i Morphological characteristics

The vegetative cells of the strain DD01 were small, rod-shaped, highly motile, colony texture glossy, white in colour (Table. 26) (Fig. 31 a and b). It showed all the characteristics of *Enterobacter* (Sneath, 1986). So, this strain was named as *Enterobacter* sp. DD01.

Table. 26 Morphological characteristics of *Enterobacter* sp. DD01

Characteristics	*Enterobacter* sp. DD01
Vegetative body structure	Rod shaped
Motility	Highly motile
Colony texture	Smooth and glossy

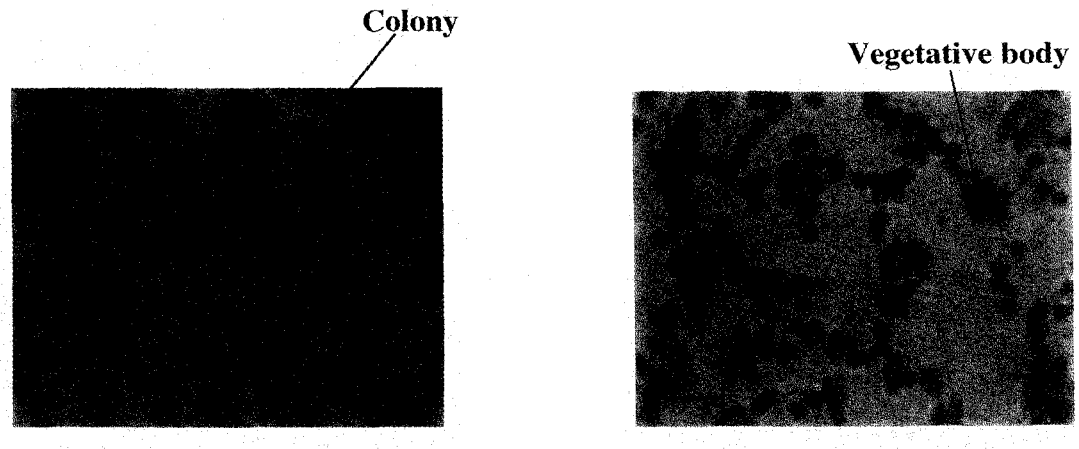


Fig. 31 a. Colonies and b. Vegetative bodies of *Enterobacter* sp. DD01 (scale 36μm)

D.b.ii Biochemical characteristics

Vegetative cells of the bacterial strain DD01 were aerobic and Gram-negative. The strain showed positive reaction for Voges Proskauer, β-galactosidase, lysine decarboxylase, ornithin decarboxylase, nitrate reduction, Simmon's citrate, esculin hydrolysis tests, but negative for phenylalanine deaminase, H_2S production, methyl red, indole, oxidase, urea hydrolysis and gelatin liquefaction tests. It utilized malonate, L-arabinose, D-xylose, D-adonitol, L-rhamnose, cellobiose, melibiose, saccharose, raffinose, trehalose, D-glucose, lactose and D-sorbitol. The bacterial strain, DD01 was therefore tentatively assigned to the genus *Enterobacter* (Table. 27).

Table. 27 Biochemical characteristics of *Enterobacter* sp. DD01

Biochemical Test	*Enterobacter* sp. DD01 reaction pattern
Gram reaction	(-)
ONPG	+
Lysine decarboxylase	+
Ornithin decarboxylase	+
Urease	-
Phenylalanine deamination	-
Nitrate reduction	+
H_2S production	-
Citrate utilization	+
V-P TEST	+
Methyl red	-
Indole	-
Malonate	+
Esculin hydrolysis	+
L-Arabinose	+
D-Xylose	+
D- Adonitol	+
L- Rhamnose	+
Cellobiose	+
Melibiose	+
Saccharose	+
Raffinose	+
Trehalose	+
D-Glucose	+
Lactose	+
D-Sorbitol	+

E. Results of bioassay

E.a Bioassay of four *Bacillus* strains

In case of *Bacillus* sp. CT01 the percent mortality of second instar *C. theivora* caterpillars varied from 61% to 77% within 9 days since inoculation/exposure. The LC_{50} value was found to be 95.50µg/ml with fiducial limits 66.729µg/ml (lower) and 124.271µg/ml (upper).

The LT_{50} values were found to be 3.75 days for 1000µg/ml, 4.11 days for 750µg/ml, 4.63 days for 500µg/ml, 6.23 days for 300µg/ml and 8.41 days for 100µg/ml concentrations.

In case of *Btk* tested on *C. theivora* larvae the LC_{50} value was found to be 436.5µg/ml with fiducial limits 402.014µg/ml (lower) and 470.986µg/ml (upper). The LT_{50} values were found to be 4.96 days for 1000µg/ml, 7.30 days for 750µg/ml and 8.23 days for 500µg/ml concentrations.

In case of *Bacillus* sp. CT02 the percent mortality of second instar *C. theivora* caterpillars varied between 55% and 76% within 9 days. The LC_{50} value was found to be 117.5µg/ml with fiducial limits 54.46µg/ml (lower) and 180.54µg/ml (upper).

The LT_{50} values were found to be 4 days for 1000µg/ml, 6.36 days for 750µg/ml, 7.5 days for 500µg/ml, 7.60 days for 300µg/ml and 7.8 days for 100µg/ml concentrations.

In case of *Bacillus* sp. CT03 the percent mortality of second instar *C. theivora* caterpillars varied between 55% and 88% within 9 days. The LC_{50} value was found to be 104.7µg/ml with fiducial limits 71.84µg/ml (lower) and 137.55µg/ml (upper).

The LT_{50} values were found to be 6.16 days for 1000µg/ml, 6.89 days for 750µg/ml, 7.4 days for 500µg/ml, 8 days for 300µg/ml and 8.6 days for 100µg/ml concentrations.

In case of *Bacillus* sp. CT04 the percent mortality of second instar *C. theivora* caterpillars varied between 54% and 78% within 9 days. The LC_{50} value was found to be 87.10µg/ml with fiducial limits 41.69µg/ml (lower) and 132.50µg/ml (upper).

The LT_{50} values were found to be 3.5 days for 1000µg/ml, 4.06 days for 750µg/ml, 5 days for 500µg/ml, 5.12 days for 300µg/ml and 8 days for 100µg/ml concentrations (Table. 28).

Table. 28 Results of bioassay of *Bacillus* sp. CT01, CT02, CT03 and CT04 strains on *C. theivora* and their comparison with *Btk*.

Name of bacterium	% mortality	LC_{50} with Fiducial Limits	LT_{50}	Hetero-geneity	Regression
Btk tested on *C. theivora*	78% for 1000 µg/ml 67% for 750 µg/ml 53% for 500 µg/ml 31% for 300 µg/ml 30% for 100 µg/ml	436.5µg/ml With 402.01 µg/ml (Lower limit) 470.98 µg/ml (Upper limit)	4.96 for 1000µg/ml 7.307 for 750µg/ml 8.238 for 500 µg/ml	$\chi^2(5)=93.8312$ for1000 µg/ml $\chi^2(5)=68.6094$ for750 µg/ml $\chi^2(5)=42.8456$ for500 µg/ml $\chi^2(5)=13.5297$ for300 µg/ml $\chi^2(5)=12.5000$ for100 µg/ml	Y=2.067X-2.236
Bacillus sp.CT01	77% for 1000 µg/ml 77% for 750 µg/ml 63 % for 500 µg/ml 62% for 300 µg/ml 61% for 100 µg/ml	95.50µg/ml With 66.72 µg/ml (Lower limit) 124.27µg/ml (Upper limit)	3.75 for 1000µg/ml 4.11 for 750µg/ml 4.63 for 500µg/ml 6.23 for 300 µg/ml 8.41for 100 µg/ml	$\chi^2(5)=44.9066$ for 100 µg/ml $\chi^2(5)=46.6477$ for 300 µg/ml $\chi^2(5)=52.0833$ for 500 µg/ml χ^2 (5)=77.3752 for 750 µg/ml χ^2 (5)=77.3752 for 1000 µg/ml	Y=3.33X-21.3
Bacillus sp. CT02	76% for 1000µg/ml 69% for 750µg/ml 63 % for 500µg/ml 59% for 300µg/ml 55% for 100µg/ml	117.5µg/ml With 54.46µg/ml (Lower limit) 180.54µg/ml (Upper limit)	4 for 1000µg/ml 6.36 for 750µg/ml 7.5 for 500µg/ml 7.60 for 300µg/ml 7.8 for 100µg/ml	$\chi^2(5)=51.187$ for 100µg/ml $\chi^2(5)=58.377$ for 300µg/ml $\chi^2(5)=66.855$ for 500µg/ml χ^2 (5)=78.576 for 750µg/ml χ^2 (5)=94.989 for 1000µg/ml	Y=0.54X+3.88
Bacillus sp. CT03	88% for 1000µg/ml 80% for 750µg/ml 75 % for 500µg/ml 68% for 300µg/ml 55% for 100µg/ml	104.7µg/ml With 71.84µg/ml (Lower limit) 137.55µg/ml (Upper limit)	6.16 for 1000µg/ml 6.89 for 750µg/ml 7.4 for 500µg/ml 8 for 300µg/ml 8.6 for 100µg/ml	$\chi^2(5)=73.972$ for 100µg/ml $\chi^2(5)=85.714$ for 300µg/ml $\chi^2(5)=120.00$ for 500µg/ml χ^2 (5)=133.333 for 750µg/ml χ^2 (5)=157.142 for 1000 µg/ml	Y=1.03X+2.90
Bacillus sp. CT04	78% for 1000µg/ml 72% for 750µg/ml 68 % for 500µg/ml 60% for 300 µg/ml 54% for 100µg/ml	87.10µg/ml With 41.69µg/ml (Lower limit) 132.50µg/ml (Upper limit)	3.5 for 1000µg/ml 4.06 for 750µg/ml 5 for 500µg/ml 5.12 for 300µg/ml 8 for 100µg/ml	$\chi^2(5)=73.972$ for 100µg/ml $\chi^2(5)=85.714$ for 300µg/ml $\chi^2(5)=103.030$ for 500µg/ml χ^2 (5)=112.500 for 750µg/ml χ^2 (5)=127.868 for 1000 µg/ml	Y=0.651X+3.73

E.b Bioassay of *Enterobacter* sp. DD01

The percent mortality of second instar *C. theiviora* caterpillars varied between 64 and 86% within 9 days. The LC_{50} value was found to be 363.1µg/ml with fiducial limits 362.94µg/ml (lower) and 363.25µg/ml (upper).

The LT_{50} values were found to be 4.61 days for 1000µg/ml, 4.96 days for 750µg/ml, 5.81 days for 500µg/ml, 5.96 for 300µg/ml and 6 days for 100µg/ml concentrations (Table. 29).

Table. 29 Results of bioassay of *Enterobacter* sp. DD01

Concentration of vegetative bodies (µg/ml)of *Enterobacter* sp. DD01	No. of tested larvae (2^{nd} instar) (n)	Actual mortality	Percentage mortality (%)	LT_{50} (days)
1000	100	86	86%	4.61
750	100	79	79%	4.96
500	100	76	76%	5.81
300	100	74	74%	5.96
100	100	64	64%	6
CONTROL	100	20	20%	-

Heterogeneity	Regression	LC_{50}	Fiducial limits
$\chi 2(5)$= 39.73 for 100µg/ml $\chi 2(5)$=58.53 for 300µg/ml $\chi 2(5)$=62.82 for 500µg/ml $\chi 2(5)$=69.62 for 750µg/ml $\chi 2(5)$=87.43 for 1000µg/ml	Y=0.69X+18.72	363.1µg/ml	362.94µg/ml (Lower limit) 363.25µg/ml (Upper limit)

F. Results of cross infectivity of the entomopathogens to other lepidopteran tea pests

It was found that all the four *Bacillus* strains and *Enterobacter* strain did not cross infect other lepidopteran tea pests other than their host.

G. Cross infectivity to beneficial lepidopteran (silk worm)

G.a Cross infectivity of *Bacillus* strains

In this experiment no notable mortality was observed due to treatment with lower as well as in higher concentrations of the *Bacillus* strains isolated from *C. theivora.*

G.b Cross infectivity of *Enterobacter* strain

In this experiment no notable mortality was observed due to treatment with lower as well as in higher concentrations of the *Enterobacter* strain isolated from *C. theivora.*

I. Field trials on biocontrol efficacy

Among the bacteria isolated from the dead and diseased leaf roller caterpillars, CT04 was marked as highly pathogenic strain. Its LC_{50} and LT_{50} values were found to be lower than the other isolated strains from *C. theivora.* Hence the strain was used for field trial which was conducted in the month of April. Four treatments (4000µg/ml, 3000µg/ml, 2000µg/ml and 1000µg/ml) with three replications for each concentration were executed along with water spray as control.

A graph of mean number of live larvae recovered after 7^{th} day against concentrations was plotted (Fig. 32). It was noted that highest concentration (4000 µg/ml) was effective in controlling the *C. theivora* population compared to other concentrations. After using the one-way ANOVA, it was found that mean number of live larvae recovered from each treatment after 7^{h} day was significantly different from each other and also from control (Table. 30). The lowest percentage of live larvae were recovered from the highest concentration sprayed. Control plots had significantly more percentage of live larvae than the treated plots. The treatment with 4000µg/ml showed the best control action even without any additives (stickers and spreaders).

Table. 30 Comparison of varying doses of *Bacillus* sp. CT04 strain on survival of leaf roller larvae on 7th day.

Treatments (µg/ml)	No. of relicate	Percent live larvae recovered after 7th day	Mean % of live larveae recovered after 7th day (Mean±SD)
4000	1 2 3	13.68 (0.378864) 11.22 (0.341566) 15.21 (0.400632)	13.37±2.01
3000	1 2 3	35.48 (0.638076) 42.04 (0.705458) 22.91 (0.49911)	33.47±9.72
2000	1 2 3	79.78 (1.104404) 87.77 (1.21353) 84.21 (1.162151)	83.92±4
1000	1 2 3	97.72 (1.41922) 88.17 (1.219678) 92.63 (1.295869)	92.84±4.77
Control	1 2 3	91.75 (1.279465) 98.91 (1.466203) 96.62 (1.385897)	95.76±3.65

F = 88.6841
p = 9.08276E-8

The means are significantly different at the 0.05 level.
Data in the parentheses were arcsine transformed values.

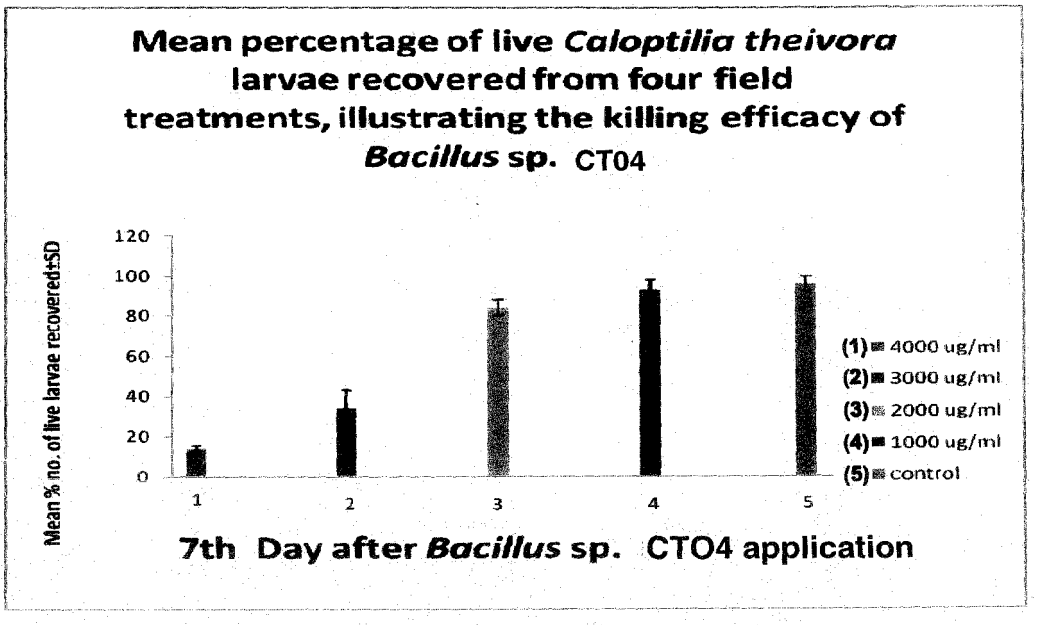


Fig. 32 Graph showing the mean percentage of live *C. theivora* larvae recovered after 7th day.

III. Red slug (*Eterusia magnifica*)

A. Symptom of bacteria infected larvae

The discoloration of the bacteria infected dead larvae were characteristic, which changed from brick red to brownish black colour with significant shrinkage of body followed by rapid decomposition. The photographs of healthy lava and a bacteria infected dead larva could be well discriminated (Fig. 33, 34).

Fig. 33 Healthy larva of *E. magnifica*

Fig. 34 Bacteria infected dead larva of *E. magnifica*

B. Mortality of *Eterusia magnifica* due to bacterial infection

Mortality of *E. magnifica* caterpillar due to bacterial infection in larval populations collected from different tea plantations of the Terai and the Dooars were studied. Mortality of red slug caterpillars collected in different months of a year was observed. As red slug (*Eterusia magnifica*) did not occur throughout the year, being an occasional tea pest, percentage mortality in population could only be determined when it was available in the tea plantation. Bacteria were found to infect almost all the populations of *E. magnifica* observed in the Terai region.

The percentage mortality in the population collected in various months of the year in the Terai region was given graphically below (Fig. 35).

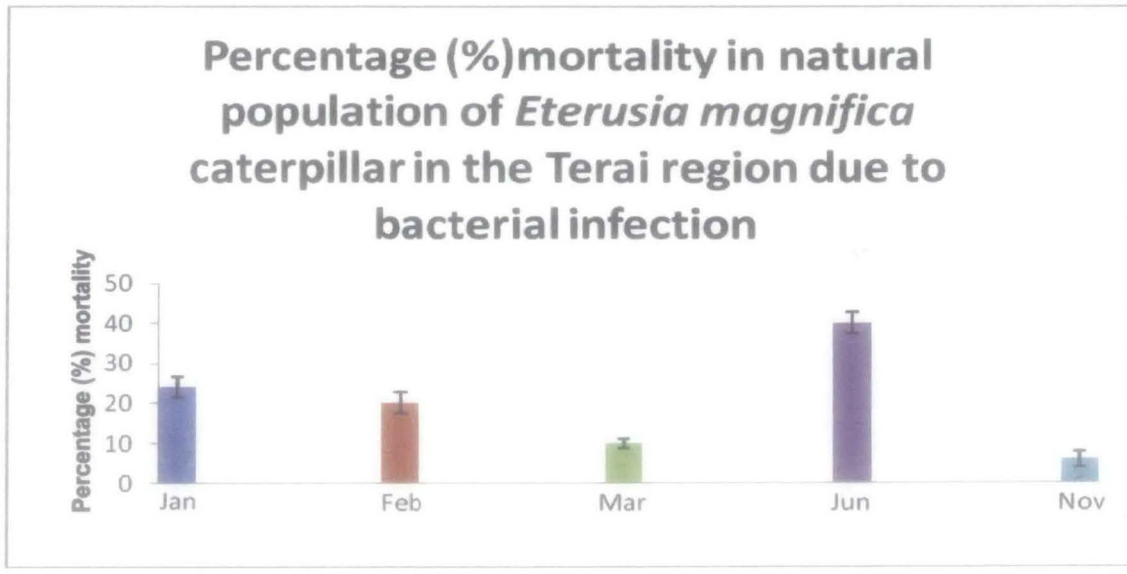


Fig. 35 Percent mortality in *E. magnifica* population due to bacterial infection the Terai region.

It was evident from the data (Fig. 35) that in January the percentage mortality was 24%, 20%, 10%, 40% and 6% in the month of Feb, Mar, June, November respectively. The percentage mortality was highest in June.

Survey was also conducted in Chuapara, Sankosh, Bhatkhawa, Mujnai, Binnaguri, Kumargram and Jiti T.E.s. of the Dooars region. The percentage mortality in *E. magnifica* population collected from the Dooars region due to bacterial infection, was 10.52% (Sankosh T.E.) and 13.04% (Chuapara T.E.).

C. A glimpse of the Bacterial strains isolated from the red slug (*Eterusia magnifica*)

For the sake of convenience in addressing, describing and discussing the various strains isolated from the red slug species, *Eterusia magnifica* in the following text, these have been given mnemonic designations.

Table. 31 Different bacterial strains isolated from cadaver of *E. magnifica* caterpillar at a glance.

Name of Tea Pest	Name of Bacteria strains isolated
Eterusia magnifica	RS01, RS02, RS03, RS04, RS05.

Five strains/isolates from *E. magnifica* were designated as RS01, RS02, RS03, RS04 and RS05 (Table. 31).

D. Preliminary characterization of Bacterial strains from *E. magnifica*

Among the five (05) isolated entomopathogenic bacterial strains the most frequently occurring entomopathogen of red slug was RS01 strain. As this strain was found to occur whole of the year it was studied in details. For the rest of the strains that occurred occasionally such only preliminary characterization and Koch's postulate were performed (Fig. 36).

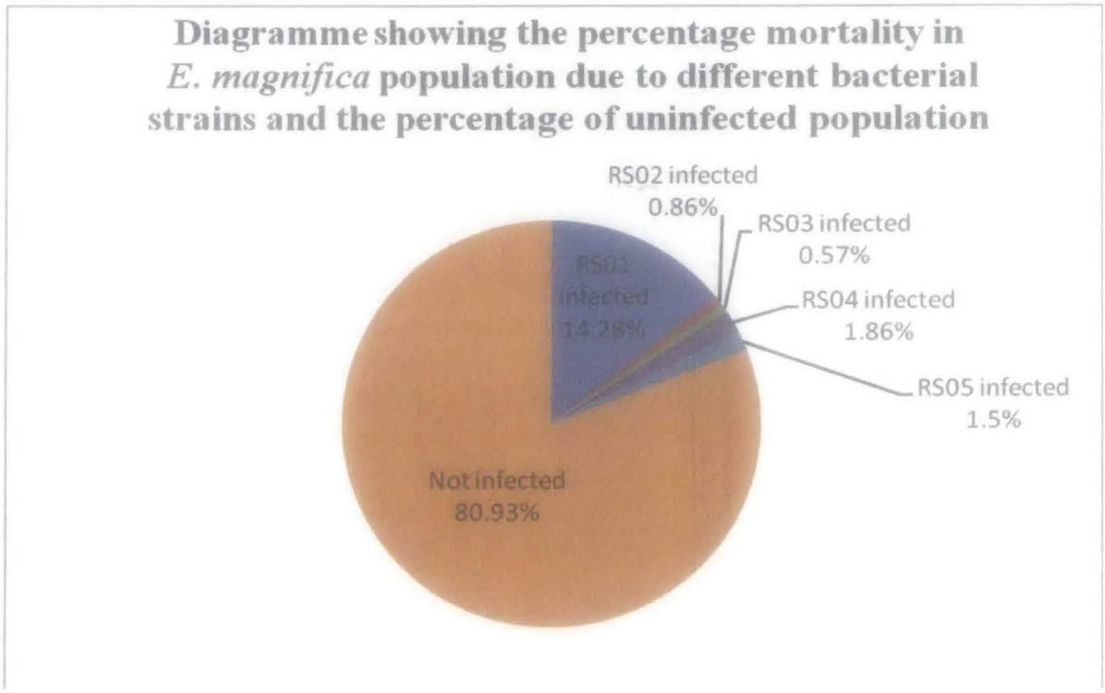


Fig. 36 Occurrence of Entomopathogenic bacterial strains in *E. magnifica* population.

D.a Morphological Characteristics

All phenotypic characteristics of the bacterial strain RS01 like cell and colony morphology, motility, shape of the endospore, crystal protein shape were found to be similar with *Bacillus thuringiensis kurstaki* (Table. 32) (Fig. 37 A, B, C, D, E, F, G). The isolate RS01 showed all the characteristics of genus *Bacillus* (Sneath, 1986) i.e. cell morphology, gram positivity, endospore formation, facultative anaerobic, catalase positive, acid production from glucose and motility.

Table. 32 Comparison of morphological characteristics of RS01 with *Btk*.

Characteristics	*Btk*	RS01
Vegetative body structure	Rod shaped and Chain like	Rod shaped and Chain like
Motility	Highly motile	Highly motile
Spore shape	Oval	Oval
Crystal protein structure	Bipyramidal	Bipyramidal

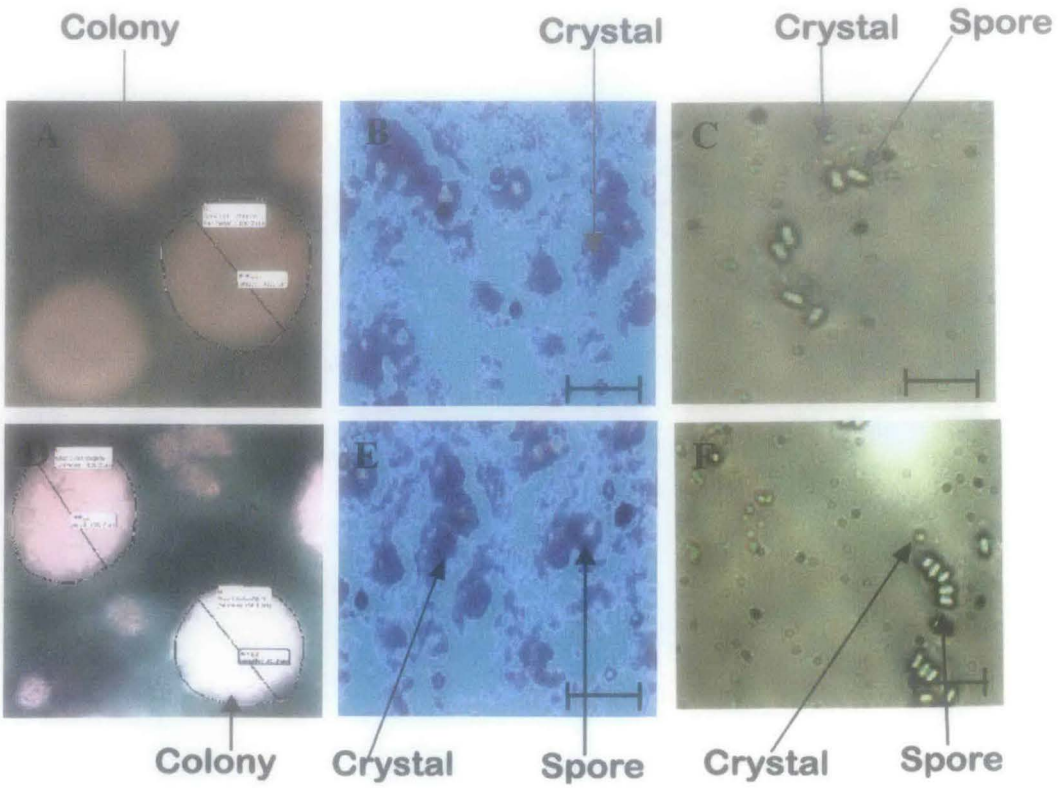


Fig. 37 A. Colony morphology, B. Crystal protein and C. Phase contrast microphotograph of spore and crystal of RS01 strain and D. Colony morphology, E. spore and crystal, F. Phase contrast microphotograph of spore and crystal of *Btk* (scale 36µm).

The other strains isolated from *E. magnifica* i.e., RS02, RS03, RS04 and RS05 also showed all the characteristics of genus *Bacillus* including cell shape, gram positivity, endospore formation, facultative anaerobic, catalase positivity, production of acid from glucose and motility (Sneath, 1986).

Since it was found that all the strains isolated from *E. magnifica* showed the characteristics of genus *Bacillus*, they were designated as *Bacillus* sp. RS01, RS02, RS03, RS04 and RS05.

D.b Biochemical Characteristics

In biochemical characteristics the RS01 strain showed positive reaction in lysine decarboxylase, ornithin decarboxylase, nitrate, urease,Voges-Proskaur and oxidase tests. It utilized trehalose and glucose. RS01 showed difference with *Btk* in ONPG, urease and oxidase tests. In utilization tests it showed difference in arabinose, xylose, cellobiose, melibiose, saccharose and lactose (Table. 33).

Table. 33 Comparative account of biochemical characteristics of *Bacillus* sp. RS01 with *Btk*.

Test	*Btk*	RS01
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	-	+

On other hand RS02 strain showed positive reaction in ornithin decarboxylase, urease, phenylalanine deamination, nitrate reduction, H_2S production, V-P, methyl red, esculin hydrolysis tests and in utilization of citrate, cellobiose, melibiose, saccharose, trehalose and glucose. It showed difference with *Btk* in ONPG, lysine decarboxylase, urease, methyl red, esculin hydrolysis and in utilization of citrate, arabinose, xylose and lactose.

RS03 strain showed positive reaction in lysine decarboxylase, ornithin decarboxylase, urease, nitrate reduction, esculin hydrolysis, methyl red tests and in utilization of citrate, malonate, cellobiose, melibiose, saccharose, trehalose and glucose. It showed difference with *Btk* in ONPG, urease, methyl red, esculin hydrolysis and in utilization of citrate, malonate, arabinose, xylose and lactose.

RS04 strain showed positive reaction in ONPG, urease, nitrate reduction, V-P, esculin hydrolysis tests and in utilization of citrate, arabinose, xylose, cellobiose, melibiose, saccharose, raffinose, trehalose, glucose and lactose. It showed difference with *Btk* in lysine decarboxylase, ornithin decarboxylase, urease, nitrate reduction, V-P and esculin hydrolysis tests and in utilization of citrate, arabinose, xylose, cellobiose, melibiose, saccharose, trehalose, raffinose, glucose and lactose.

RS05 strain showed positive reaction in ONPG, nitrate reduction, V-P, H_2S and esculin hydrolysis tests and in utilization of citrate, malonate, xylose, cellobiose, melibiose, trehalose, raffinose, saccharose, glucose and lactose. It showed difference with *Btk* in lysine decarboxylase, ornithin decarboxylase, H_2S and esculin hydrolysis tests and in utilization of citrate, malonate, arabinose and raffinose (Table. 34).

Table. 34 Comparison of biochemical characteristics of bacterial strains (RS02, RS03, RS04 and RS05) with *Btk*.

Sl. No.	Name of Biochemical tests	*Btk*	RS02	RS03	RS04	RS05
1.	ONPG	+	-	-	+	+
2.	Lysine decarboxylase	+	-	+	-	-
3.	Ornithin decarboxylase	+	+	+	-	-
4.	Urease	-	+	+	+	-
5.	Phenylalanine deamination	-	-	-	-	-
6.	Nitrate reduction	+	+	+	+	+
7.	H_2S production	-	-	-	-	+
8.	Citrate utilization	-	+	+	+	+
9.	V-P Test	+	+	+	+	+
10.	Methyl red	-	+	+	-	-
11.	Indole	-	-	-	-	-
12.	Malonate	-	-	+	-	+
13.	Esculin hydrolysis	-	+	+	+	+
14.	Arabinose	+	-	-	+	-
15.	Xylose	+	-	-	+	+
16.	Adonitol	-	-	-	-	-
17.	Rhamnose	-	-	-	-	-
18.	Cellobiose	+	+	+	+	+
19.	Melibiose	+	+	+	+	+
20.	Saccharose	+	+	+	+	+
21.	Raffinose	-	-	-	+	+
22.	Trehalose	+	+	+	+	+
23.	Glucose	+	+	+	+	+
24.	Lactose	+	-	-	+	+
25.	Oxidase	-	-	-	-	-

D.c Growth characteristics: determination of generation time

The doubling time was 120 min in case of RS01 strain and 42 min in case of *Btk* (Table. 35).

Table. 35 Comparative account of doubling time of *Bacillus* sp. RS01 and *Btk*.

Name of Bacterial strains	Doubling time
***Bacillus* sp. RS01**	**120 mins.**
Btk	**42 mins.**

The doubling time was 84 min in case of RS02, 72 min in case of RS03, 30 min in case of RS04 and 42 min in case of RS05 strains (Table. 36).

Table. 36 Comparative account of doubling time of *Bacillus* strains (RS02, RS03, RS04 and RS05) and *Btk*.

Name of Bacterial strains	Doubling time
Bacillus sp. RS02	84 mins.
Bacillus sp. RS03	72 mins.
Bacillus sp. RS04	30 mins.
Bacillus sp. RS05	42 mins
Btk	42 mins.

D.d SDS-PAGE profile of crystal protein

SDS-PAGE analysis of the crystal protein content of RS01 strain showed two main protein bands (53 and 49 kDa) where as in *Btk* 52 kDa molecular weight protein band was present. So, differences in crystal protein profile was observed between RS01 strain and *Btk* (Fig. 38).

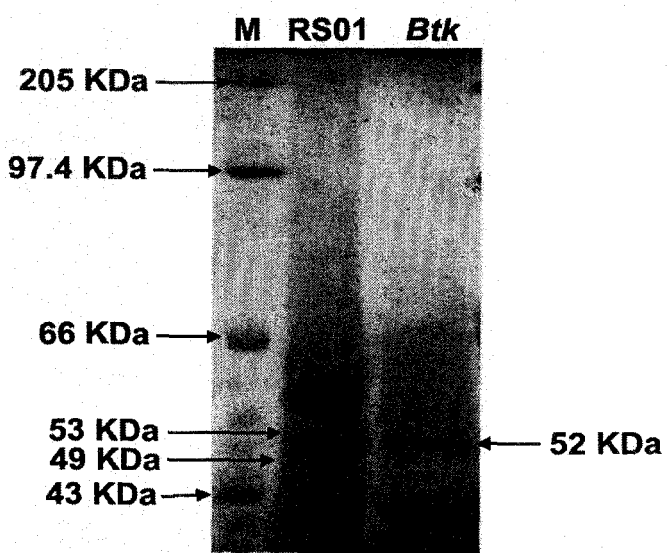


Fig. 38 SDS-PAGE profile of crystal protein of *Bacillus* sp. RS01 and *Btk*

D.e Qualitative (SDS-PAGE) analysis of whole body protein of bacterium

Difference in banding pattern of whole body protein profile existed between *Btk* and RS01 strain. One protein band of 31 kDa present in *Btk*, was absent in RS01 strain. Instead in RS01 strain a 34 kDa protein band was present which was totally absent in *Btk*. So, major differences were evident between RS01 and *Btk* strain (Fig. 39).

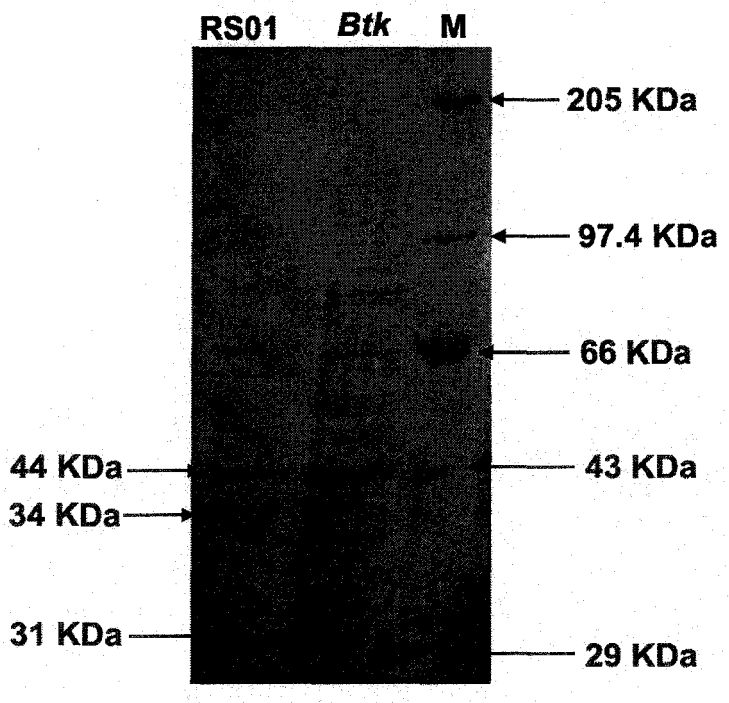


Fig. 39 SDS-PAGE profile of whole body protein of *Bacillus* sp. RS01 and *Btk*.

E. Results of bioassay

The percent mortality of second instar *E. magnifica* caterpillars varied between 20 and 78% within 9 days. The LC_{50} value was found to be 458.2µg/ml with fiducial limits 457.95µg/ml (lower) and 458.24µg/ml (upper).

The LT_{50} values were found to be 5.6 days for 1000µg/ml, 5.69 days for 750µg/ml and 6.19 days for 500µg/ml concentrations. In case of *Btk* which was tested on *E. magnifica* larvae, the LC_{50} value was found to be 416.9µg/ml with fiducial limits 416.76µg/ml (lower) and 417.034µg/ml (upper). The LT_{50} values were found to be 6.67 days for 1000µg/ml, 6.93 days for 750µg/ml and 7.54 days for 500µg/ml concentrations (Table. 37).

Table. 37 Results of bioassay of *Bacillus* sp. RS01 compared with *Btk*.

Name of bacterium	% mortality	LC_{50} with Fiducial Limits	LT_{50}	Hetero-geneity	Regression
Btk tested on *E. magnifica*	87% for 1000µg/ml 83% for 750µg/ml 77% for 500µg/ml 21% for 300µg/ml 20% for 100µg/ml	416.9µg/ml With 416.76µg/ml (Lower limit) 417.034µg/ml (Upper limit)	6.67 for 1000µg/ml 6.93 for 750µg/ml 7.54 for 500 µg/ml	$\chi^2(5)$=118.6868 for 1000µg/ml $\chi^2(5)$=107.1048 for 750µg/ml $\chi^2(5)$=91.3234 for 500µg/ml $\chi^2(5)$=4.6192 for 300µg/ml $\chi^2(5)$=3.9216 for 100µg/ml	Y=2.61X-8.81
Bacillus sp. RS01	78% for 1000µg/ml 72% for 750µg/ml 61 % for 500µg/ml 21% for 300µg/ml 20% for 100µg/ml	458.2µg/ml With 457.955 µg/ml (Lower limit) 458.245 µg/ml (Upper limit)	5.6 for 1000µg/ml 5.69 for 750µg/ml 6.19 for 500µg/ml	$\chi^2(5)$= 93.8312 for 1000 µg/ml $\chi^2(5)$=79.4543 for 750 µg/ml $\chi^2(5)$=56.7966 for 500µg/ml $\chi^2(5)$=4.6192 for 300µg/ml $\chi^2(5)$=3.9216 for 100µg/ml	Y=2.16X-4.16

F. Results of cross infectivity to other lepidopteran tea pests

It was found that *Bacillus* sp. RS01 did not affect other lepidopteran tea pests other than their host.

G. Result of cross infectivity to beneficial lepidopteran (silk worm)

In this experiment no notable mortality was observed due to treatment with lower as well as in higher concentrations of the *Bacillus* strain isolated from *E. magnifica*.

H. Field trials on biocontrol efficacy

Among the bacteria isolated from the dead and diseased red slug caterpillars, the one i.e. RS01 was a highly pathogenic strain. Field trial was conducted in the month of September. Four treatments (12000µg/ml, 11000µg/ml, 10000µg/ml and 9000µg/ml) with three replications for each concentration were executed along with water spray as control.

A graph of mean percentage of live larvae recovered after 3rd, 5th and 7th day was plotted (Fig. 40 A, B, C). From the graph it was evident that the highest concentration (12000µg/ml) was effective in controlling *E. magnifica* population compared to other concentrations. After analyzing the one-way ANOVA, it was found that mean percentage of live larvae recovered from each treatment after 7th day were significantly different from each other and also from control (Table. 38 A, B, C). The lowest percentage of live larvae were recovered from the highest concentration sprayed. Control plots had significantly more percentage of live larvae than the treated plots. The treatment with 12000µg/ml showed the best control action.

Table. 38 A. Comparison of varying doses of *Bacillus* sp. RS01 strain on survival of red slug larvae on 7th day.

Treatments (µg/ml)	No. of relicate	Percent live larvae recovered after 7th day	Mean % of live larvae recovered after 7th day (Mean±SD)
12000	1	8.23 (0.290968)	10.98±2.76
	2	10.97 (0.33758557)	
	3	13.75 (0.379881)	
11000	1	32.14 (0.602764)	34.89±2.40
	2	35.95 (0.6429802)	
	3	36.58 (0.649532)	
10000	1	88.23 (1.220608)	89.18±1.32
	2	88.63 (1.22686126)	
	3	90.69 (1.260729)	
9000	1	91.25 (1.270499)	92.47±4.57
	2	88.63 (1.22686126)	
	3	97.53 (1.41298)	
Control	1	95.23 (1.350618)	94.26±5.08
	2	98.79 (1.46057328)	
	3	88.76 (1.228914)	

F = 121.2213

p = 1.98725E-8

The means are significantly different at 0.05 level.

Data in the parentheses were arcsine transformed values.

38 A

38 B. Comparison of varying doses of *Bacillus* sp. RS01 strain on survival of red slug larvae on 5^{h} day.

Treatments (µg/ml)	No. of relicate	Percent live larvae recovered after 5th day	Mean % of live larvae recovered after 5th day (Mean±SD)
12000	1 2 3	88.23 (1.220608) 95.12 (1.348052) 96.25 (1.375916)	93.2±4.34
11000	1 2 3	83.33 (1.150217 82.02 (1.132908) 81.7 (1.128756)	82.35±0.86
10000	1 2 3	88.23 (1.220608) 89.77 (1.245232) 95.34 (1.353213)	91.11±3.74
9000	1 2 3	93.75 (1.318116) 89.77 (1.245232) 98.76 (1.45921)	94.09±4.50
Control	1 2 3	95.23 (1.350618) 78.79 (1.092189) 93.25 (1.307973)	89.09±8.97
F = 2.16455 p = 0.14683 --- The means are NOT significantly different at 0.05 level. Data in the parentheses were arcsine transformed values.			

38B

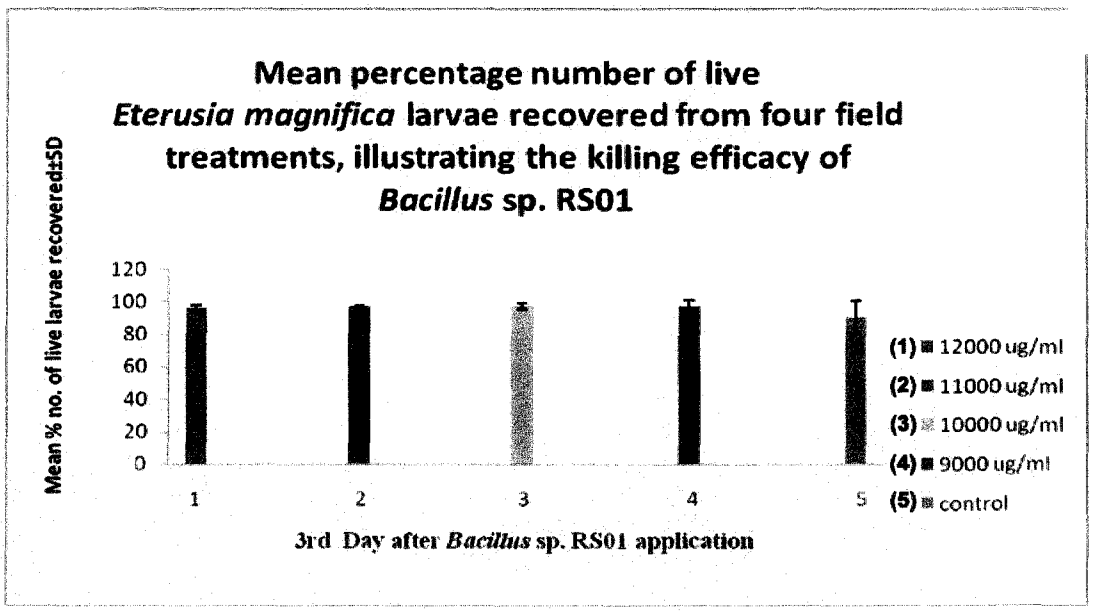
38 C. Comparison of varying doses of *Bacillus* sp. RS01 strain on survival of red slug larvae on 3rd day.

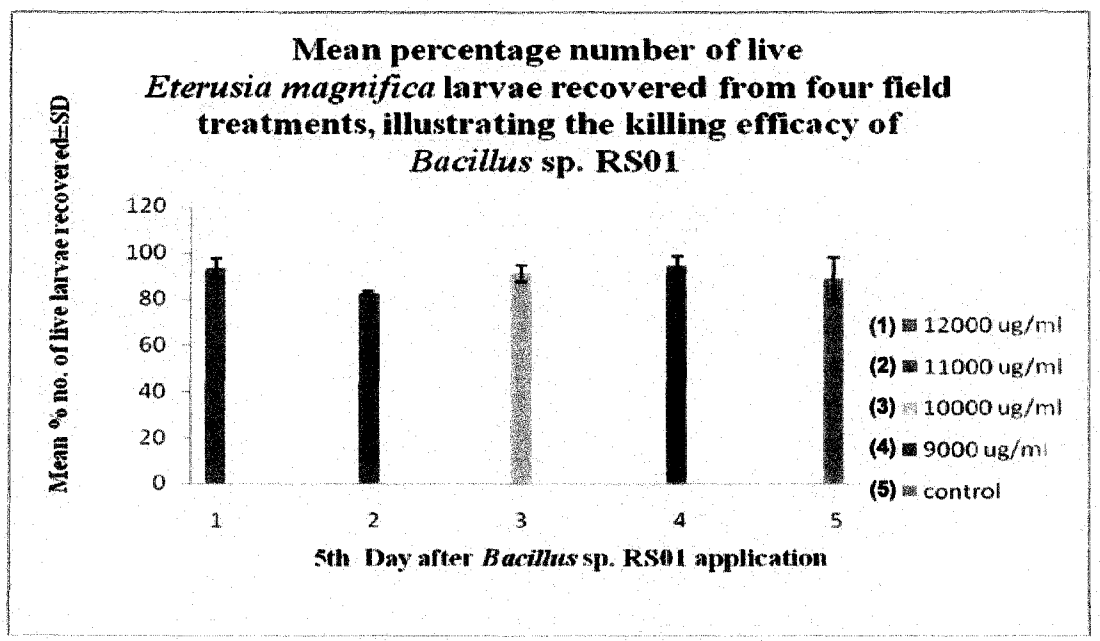
Treatments (µg/ml)	No. of replicate	Percent live larvae recovered after 3rd day	Mean % of live larvae recovered after 3rd day (Mean±SD)
12000	1	92.99 (1.302837)	95.6±2.35
	2	97.56 (1.413949)	
	3	96.25 (1.375916)	
11000	1	95.23 (1.350618)	96.09±1.27
	2	95.5 (1.35704)	
	3	97.56 (1.413949)	
10000	1	97.64 (1.416563)	97.28±1.78
	2	98.86 (1.463822)	
	3	95.34 (1.353213)	
9000	1	98.75 (1.458759)	97.31±3.63
	2	93.18 (1.306582)	
	3	100 (1.570796)	
Control	1	98.8 (1.461032)	90.28±10.33
	2	78.79 (1.092189)	
	3	93.25 (1.307973)	

F = 0.89028
p = 0.50431

The means are NOT significantly different at 0.05 level.
Data in the parentheses were arcsine transformed values.

38 C





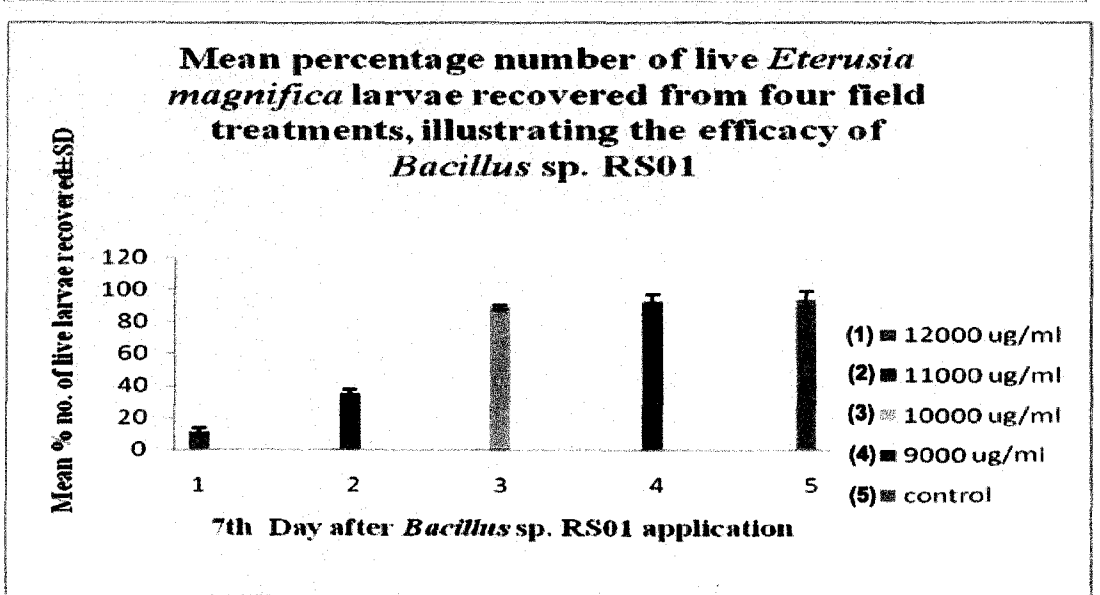


Fig. 40 A, B, C Graphs showing the percentage live larvae recovered after 3rd, 5th and 7th day after treatment with entomopathogenic *Bacillus* sp. RS01.

6

Discussion

6. DISCUSSION

On occurrence of entomopathogenic bacteria of lepidopteran pests in Terai-Dooars tea plantations

Caterpillars of the lepidopteran tea pests, *Buzura suppressaria*, *Hyposidra talaca*, *Caloptilia theivora* and *Eterusia magnifica* after being collected from the natural populations of Sub Himalayan tea plantations were reared in laboratory. The number of larvae dying during first few days of rearing, with symptoms of bacterial infection (i.e. characteristically blackish with significant shrinkage of body and discolouration followed by rapid decomposition) as has been observed by earlier authors (Lacey and Brooks, 1997; Shimanuki and Knox, 1991; Bach, 1985) gave an indirect estimate of the proportion of the population that bore bacterial infection in field (plantation) (Leucona, 1996; Meca *et al.*, 2009). The larvae dying by force killing did not show such symptoms after 12 hour incubation compared to the bacteria infected larvae after same period of time. In a similar study soil-dwelling noctuids, mostly cutworm larvae *Agrotis segetum* were collected from various arable crops, from 39 sites in England and Wales between 1975 and 1978 and were individually reared in the laboratory. Of these that died (4·8%) were found infected with entomopathogens, thus giving an estimate of the proportion of naturally infected population. The possibility of controlling cutworms with their pathogens has been recommended in this study (Sherlock, 2008).

Large scale larval mortality in all the concerned species of tea lepidopterans due to bacterial infection occurred in the rainy season from June to September with a high average rainfall in Himalayan Terai region. Correlation between increased rainfall and high larval mortality in lepidopteran pests was also evident in earlier studies (Surtees, 1971; Agrios, 2005). The main cause assigned for such an enhanced bacterial infection is due to quick transmission of the entomopathogen by rain water. Moreover, most bacterial diseases are particularly favored by high moisture or high relative humidity. Bacteria multiply faster and are more active during wet weather. Bacterial infection causes damage to the tissue of the larvae, which in cadavers help release greater numbers of bacteria through tissue disintegration, as such causing more of infection in the wet weather conditions (Surtees, 1971; Agrios, 2005).

The natural bacteriosis of lepidopteran insects leading to epizootics and mass mortality of natural populations is supported by several other works. According to the works of Osborn *et al.*, 2002, the larvae of *Hylesia metabus* Crammer (Lepidoptera: Saturniidae) were susceptible to several pathogens indigenous to the area in which they were found. Some larvae showed characteristic symptoms of bacterial infection; they became flaccid and lethargic, and showed a marked loss of appetite. They isolated and identified 29 bacterial strains from live, dead and experimentally infected *H. metabus* larvae, and evaluated their pathogenic activity. The bacteria which principally caused mortality in the larvae were identified as: *Pseudomonas aeruginosa* (60–93.3%), *Proteus vulgaris* (20%), *Alcaligenes faecalis*, *Planococcus* sp. and *Bacillus megaterium* (10%).

In India, infestation of rice by leaf folders (LFs) viz. *Cnaphalocrocis medinalis, Marasmia exigua* and *Brachmia aurotrea* cause up to 60% yield losses. Among the three LFs, *C. medinalis* emerged as the major and regular pest of Rabi rice (grown during December to May under irrigated conditions) in India. A few natural bacterial (*Bacillus thuringiensis*) pathogens were found to be effective against the LFs both in the field and net house conditions. Interactions of the important factors were analysed to understand their significance in outbreaks of natural epizootics of the bacteria (Danger, 2008).

The development of an epizootic was studied in a dense population of larvae of the gypsy moth, *Porthetria dispar*. One of the two pathogens involved was a variant of *Streptococcus faecalis*. The behavior of the larvae increased the relative density of the population and 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, forming an abundant source of inoculum for feeding larvae. This occurred early enough to account for the massive increase in disease. Frequency of cadavers encountered in the field and disease development in 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 instars. Earlier observations substantiated that the epizootic was density-dependent and that the rapid spread of pathogens in the susceptible population was enhanced by the behavior of the larvae during the early instars (Doane, 1970).

High mortality records in population, of *B. suppressaria*, *H. talaca*, *C. theivora* and *E. magnifica* collected from tea plantations especially in rainy season give a clear indication of the presence of naturally occurring entomopathogenic bacteria in all the concerned tea pests. For effective utilization of the bioagents for the regulation of the pest population, it is very important that the influence of biotic and abiotic factors on the incidence, spread and sustenance of the insect pathogens and their 'coincidental' ecology must be well understood. In addition to this, their interactive patterns need to be determined so that accurate decisions regarding manipulation of the pathogens may be contemplated for integrated management of the pest (Gopal *et al.*, 2002).

The fall armyworm *Spodoptera frugiperda* was susceptible to atleast 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).

Characterization of the entomopathogenic bacteria

Morphological characteristics, biochemical characteristics and growth characteristics

All the purified *Bacillus* strains isolated from *B. suppressaria*, *H. talaca*, *C. theivora* and *E. magnifica* were rod shaped, endospore producing, gram positive and facultative anaerobes. They were catalase positive and acid producing from glucose (Sneath, 1986). The *Bacillus* strains showed typical characteristics of *Bacillus thuringiensis* (*Bt*) in their cell morphology and crystal production during sporulation. Based on crystals, the distinguishing characteristic of *Bt* (Heimple and Angus, 1958), the isolates were identified as *Bt* strains (Bai *et al.*, 2002; Brussock and Currier, 1990). Patel *et al.* in 2009 and Halt *et al.* in 1994 identified some isolates of *Bacillus* as *Bacillus thuringiensis* on the basis of morphological and biochemical characteristics after Bergey's "Manual of Determinative Bacteriology" and other standard literatures. Entomopathogenic *Bacillus* from Simulium larvae and adults were also identified and characterized using the same literature (Cavados *et al.*, 2001).

The crystal protein shape of the bacterial strains was found to be oval in case of BS01, HT01, HT02 and CT02, pyramidal in case of CT01 and bipyramidal in case of CT03, CT04 and RS01 strains. On the other hand the shape of the crystal protein of

Bacillus thuringiensis kurstaki (*Btk*) is bipyramidal. So, from this data it can be said that except CT03, CT04and RS01 strains all the other strains showed dissimilarity with *Btk* in respect of crystal protein shape. Such variation in crystal protein shape is reported by several authors with deviation from regular or common bipyramidal shape (Aronson *et al.*, 1986; Kati *et al.*, 2007; Kati *et al.*, 2007b; Lopez-Meza and Ibarra, 1996; Ibarra *et al.*, 2003).

In another finding, the non-sporulating bacterial strain isolated from *C. theivora* showed all the phenotypic characteristics of *Enterobacter* sp. such as small rod shaped cell, white, glossy and circular colony. It displayed 100% similarity with physiological characteristics of the genus *Enterobacter* (Sneath, 1986b) but was unique as a new find from the leaf roller, *C. theivora*, hence was designated as DD01 strain of genus *Enterobacter*.

All the *Bacillus* strains (*Bacillus* sp. BS01, *Bacillus* sp. HT01, *Bacillus* sp. HT02, *Bacillus* sp. CT01, *Bacillus* sp. CT02, *Bacillus* sp. CT03, *Bacillus* sp. CT04, and *Bacillus* sp. RS01) isolated, showed few but marked differences in biochemical phenotype among themselves as well with *Btk*. With respect to the differences scored from biochemical and physiological phenotype, the isolates were distinguished as different strains of *Bacillus* sp. On the other hand, the strain DD01 being gram negative, aerobic, motile and showing positive reaction in Simmon's citrate test, Voges Proskauer, β-galactosidase, lysine decarboxylase, ornithin decarboxylase and nitrate reduction tests was characterized as one belongs to *Enterobacter* sp. Further from the results of utilization tests of malonate, L-arabinose, D-xylose, D-adonitol, L-rhamnose, cellobiose, mellibiose, saccharose, raffinose, trehalose, D-glucose, lactose and D-sorbitol, its determination as a strain of *Enterobacter* sp. was confirmed.

The method of characterization of entomopathogenic bacteria with the aid of morphological, physiological and biochemical tests was also evident in the works of Orduz *et al.*, 1996; Aslim *et al.*, 2002; Kati *et al.*, 2007a; Kati *et al.*, 2007; Bai *et al.*, 2002; Tyrell *et al.*, 1981.

There are some morphological and biochemical ways of recognizing or differentiating strains before considering other biological differences perhaps more important to epizootiology. Differentiating techniques of strains depend on the type of micro-organisms. There is a variety of determining methods available for entomopathogenic bacteria, including biochemical analysis (Fuxa and Tanada, 1987).

Growth studies with the concerned *Bacillus* strains showed differences in generation times among them and with *Btk* as well. Such differences in growth physiology can also be an added phenotypic differentiating tool for the *Bacillus* strains and *Btk*. The characterization of bacteria with the help of growth characteristics is supported by the works of Kashyap and Amla, (2007).

Crystal and whole body protein profile

An analysis of solubilized crystal protein profiles of all the *Bacillus* strains (BS01, HT01, HT02, CT01, CT02, CT03, CT04 and RS01) on SDS-PAGE indicated differences in banding pattern among themselves and with that of *Btk*.

It is an important approach to search for novel insecticidal proteins which may help control lepidopteran pests. Cry protein appears to be in concordance with the toxicity. SDS-PAGE analysis suggested that in almost all the cases, the bacterial isolates produced different molecular weight proteins different from that of *Btk*. This may also be responsible for higher activity (Patel *et al.*, 2009). Here in the present work detection of 53 kDa and 49 kDa in RS01 strain, 51 kDa in CT01 strain, 37 kDa and 31 kDa in CT02 strain, 118 kDa in CT03 strain, 38 kDa and 29 kDa in CT04 strain, 86 kDa and 40 kDa in HT01 strain and 92 kDa, 76 kDa, 64 kDa, 38 kDa, 30 kDa, in case of HT02 *Bacillus* strains established their distinction and separate identity that was not present in *Btk* strain.

It is known that the crystal protein dissolves in the gut of susceptible larvae releasing one or more insecticidal proteins (endotoxins) of 27-140 kDa range (Charnley, 1991). The SDS-PAGE analysis of the isolated *Bacillus* strains from the lepidopteran pests of tea showed presence of several major polypeptides. SDS-PAGE analysis of crystal proteins gives the idea about their size and types of cry genes. For example, two distinct major bands of 130 kDa and 60 kDa proteins were predicted to be of size range of Cry1 and Cry9 type of insecticidal proteins by Patel *et al.*, (2009); Crickmore *et al.*, (1998). Many Cry proteins fall in this range the profile of crystal protein in SDS-PAGE is useful for characterization of delta-endotoxin families (Cavados *et al.*, 2001). Finally PCR based analysis is needed to confirm the type of Cry protein (Patel *et al.*, 2009). However Zhu *et al.*, (2009) has shown the presence of cry protein on SDS-PAGE without application of PCR.

SDS-PAGE profile of crystal proteins have been of major importance in discriminating bacterial strains (Ibarra *et al.*, 2003; Lopez-Meza and Ibarra, 1996; Kati *et al.*, 2007). Whereas the total cell protein profile has been helpful as additional diagnostic tool for comparing bacterial strains (Costas, 1990; Costas, 1992). Protein profile of whole cell can differentiate organisms up to species level, but for *Bacillus* species it can also differentiate up to subspecies level (Berber, 2004). Here the whole body protein profiles of all the newly isolated strains in question, except *Bacillus* sp. BS01, CT02 and RS01 have shown similar banding pattern to *Btk*. Hence, it may be concluded that BS01, CT02 and RS01 are different from *Btk* implying diversity amongst crystal protein bearing *Bacillus* strains. Though HT01, HT02, CT01, CT03 and CT04 strains have shown identical protein profile but their difference in some biochemical parameters, crystal protein profile, morphology of crystal protein etc. also proclaim their distinctions from *Bacillus thuringiensis kurstaki* (*Btk*).

Strains that differ in some properties or others have commonly been observed in an entomopathogen species. There are even some examples of different strains being tested in an insect's habitat for microbial control; such examples permit conclusion relating to epizootiology (Fuxa and Tanada, 1987). One of the most important differences among strains is in their pathogenicity. The best studied differences among strains are in their virulence. The strain differences were pertinent in terms of virulence, in relation to toxin production, particularly in *B. thuringiensis*. Strain differences in morphology or life cycle have been well documented in bacteria (Fuxa and Tanada, 1987).

A complete characterization of *Bacillus* strains with phylogenetic consideration would have been more appreciable. But the proposal is not considered under the purview of present investigation as it deals with other aspects of applied dimensions. Determination of the composition and toxicity of the parasporal crystals, by means of SDS-PAGE analysis and bioassay, is also a useful complement for gene identification (Patel *et al.*, 2009). Although it was found that the newly found bacterial strains (BS01, HT01, HT02, CT01, CT02, CT03, CT04 and RS01) were to some extent similar with *Bacillus thuringiensis kurstaki* yet more detailed investigations at the genetic level is required for confirming differences at the species and strain level of classification.

Bioassay

The infectivity and the killing efficacy of the bacterial strains (BS01, HT01, HT02, CT01, CT02, CT03, CT04, DD01 and RS01) were determined through bioassay on the concerned pests. Before going for field study it was necessary to determine the toxicity in laboratory condition. The test conducted in laboratory for a "spore and crystal producing bacterium", only with purified crystals may not be actual representation of the toxicity of a strain under natural conditions. As such, bioassays with spore-crystal mixtures appear more appropriate both under laboratory and field condition with a comparison of the activity of commercialized strains such as *Btk* to determine the exact potential of the strain (Itoua-Apoyola *et al.*, 1995).

In some insect hosts at high pH of the gut bacterial spores to germinate resulting in increase in numbers of bacteria. The bacteria invades and multiply in the body. When the body contents can support no more bacteria, spores are formed, allowing the spores to get released by disintegrating which survive in environment until getting into (inoculating) healthy larvae (Burges, 2001). In bioassay tests, the spore–crystal mixture has a higher larvicidal activity as had been reported by Yaman *et al.*, (2002) and Johnson *et al.*, (1998). They established that addition of spores to delta-endotoxin was essential to induce significant mortality in larvae of *Chrysomya albiceps*.

In the present study leaf dip method was used in laboratory bioassay. 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; Martinez *et al.*, 2004).

In all the cases it was found that the LC_{50} value of BS01, HT01, HT02, CT01, CT02, CT03 and CT04 were comparatively lower than the commercial used biopesticide, i.e. *Btk* for lepidopteran caterpillars. The *Enterobacter* sp. DD01 was found to be highly pathogenic to *C. theivora* as indicated by its low LC_{50} and LT_{50} values. Both low LC_{50} and LT_{50} values proclaimed a higher toxicity of most of the newly found bacterial strains in question. Although the LC_{50} value was slightly higher than *Btk* in case of RS01 strain but the LT_{50} values of all its concentration were lower compared to *Btk*. It is reported that the estimated LT_{50} decreased with the increase in

dose (Trang and Chaudhury, 2002). LT_{50} values provide additional information that pathogen that kills quickly, thus reducing crop loss help reduce damage due to pest attack (Kadir, http://www.avrdc.org/pdf/90dbm/90DBM21.pdf). It has been found that two insecticides with similar LC_{50} but one with a low LT_{50} values gives the lower one more effectivity as it requires less time to kill the insect (Ahmad *et al.*, 2005).

Natural bacterial isolates had 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 of higher insecticidal potential than that of reference strain (Kati *et al.*, 2007; Bai *et al.*, 2002).

Therefore, after considering all data from available reports it may be inferred that most of the newly reported bacterial strains have the potential to be developed in future as microbial biopesticides if not better than *Btk* which is already in use for controlling different lepidopteran tea pests.

Cross infectivity to other harmful lepidopteran larvae

Cross infectivity of an entomopathogenic strain of bacterium isolated from a pest species to the same or other groups of insects is important for controlling more than one insect pest which are harmful to the same tea plantation. It is a matter of fact that lepidopteran group of insects cause a substantial crop loss every year. Recently, *B. suppressaria*, *H. talaca*, *C. theivora* and *E. magnifica* caterpillars have gained greater economic importance as regular lepidopteran pests of tea in North-East India, including the Darjeeling foothill region. The bacterial strains which were isolated from these tea pests were no doubt found to be potential candidates as microbial bio-agents for controlling the host lepidopteran. Moreover, in the cross-infectivity experiment it was found that among all the isolated bacterial strains only BS01, HT01 and HT02 have the ability to infect and cause mortality to other species of tea pests (other than the pest from which they had been isolated). On one hand the strain BS01 originally isolated from *B. suppressaria* infected *H. talaca* and *C. theivora* while on the other hand HT01 and HT02 originally isolated from *H. talaca*, could infect *B. suppressaria* and *C. theivora* caterpillars.

Different literature have indicated the consequences of exposure of non-target organisms to *Bt*, (WHO, 1982; Lacey and Mulla, 1990; Melin and Cozzi, 1990; Molloy, 1992; Otvos and Vanderveen, 1993). A strain pathogenic to three or four host

species in a particular habitat is likely to produce greater numbers of infectious units and more frequent or severe epizootics (Fuxa and Tanada, 1987). Martinez *et al.*, (2004) showed that strain HU4-2 exhibited a high toxicity towards both *H. armigera* and *Spodoptera* spp. As these species of Lepidoptera were not controlled efficiently by a single *Bt* based biopesticide, the wide host range of strain HU4-2 made it a potentially useful candidate for the combined biological control of these important pests (Martinez *et al.*, 2004).

The present finding on cross infectivity appears to be very useful one for controlling the three insect pests (*B. suppressaria*, *H. talaca* and *C. theivora*) of tea with the help of any one of the bacterial strain i.e., *Bacillus* sp. BS01 or *Bacillus* sp. HT01 or *Bacillus* sp. HT02. Different formulations could be developed in future by combining any two or three of the said bacterial strains for effective in reductions of the pest infestation.

Cross infectivity of the bacterial strains to silk worm

The bacterial isolates from infected larvae, strain BS01 from *B. suppressaria*, strains, HT01 and HT02 from *H. talaca*, strains, CT01, CT02, CT03, CT03 and DD01 from *C. theivora* and strain RS01 from *E. magnifica* were found to be potential microbial entomopathogens for the concerned tea pests. But before going in to field study or field spray it was necessary to determine their toxicities to beneficial lepidopteran insects which might be at risk after spraying in the field. As the silk worm industry is running side by side with tea industry in northern West Bengal, cross-infectivity of the isolated bacterial strains on silk worm were tested. The larvae of silkworm *Bombyx mori* are useful not only as an animal model to study infections by bacteria or fungi that are pathogenic (Hussain *et al*, 2006). Early second instar larvae of multivoltine silk worms (*Bombyx mori nistari*), 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 caterpillar, however the harmful effect of the bacteria on silk worm needs confirmation (Mukherjee and Singh, 1993).

In the present study it was found that the all the strains of bacteria isolated from *B. suppressaria*, *H. talaca*, *C. theivora* and *E. magnifica* were not infecting the

silk worm larvae. Despite *per os* bacterial exposure through mulberry leaf feeding there was no mortality in the silk worm. So, from these data we can say that the newly isolated bacterial strains, BS01, HT01, HT02, CT01, CT02, CT03, CT04, DD01 and RS01 are apparently safe for spraying in the tea plantation for controlling the respective pests. Any consequential damage to beneficial insects specially silk worm industry in particular is ruled out. Also, from literature it is 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 pest insects, are relatively harmless to silk worms (Khetan, 2001).

Nevertheless, 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 resisted given the diversity of available *Bt* strain.

Field study

Biological insecticides of natural origin have low mammalian and environmental toxicity which are important characteristics to be considered when managing pests in with sound environmental approach (Guerrero *et al*, 2007).

Field study of the isolated strains of entomopathogens was necessary to know their potential of killing their natural hosts (=pest) in natural environmental condition (tea plantations) because environmental parameters play a major role in sustenance of the toxicity of the entomopathogen in field condition. Among the most frequently found three *Bacillus* strains (*Bacillus* sp. BS01, *Bacillus* sp. HT01 and *Bacillus* sp. HT02) from looper caterpillar (*B. suppressaria* and *H. talaca* respectively), the killing potential of *Bacillus* sp. HT01 strain was quite high in laboratory experiments. A satisfactory result was obtained on its spraying in the plantation too, with a substantial reduction in looper population. Among the most frequently found bacterial strains of *C. theivora*, *Bacillus* sp. CT01, *Bacillus* sp. CT02, *Bacillus* sp. CT03 and *Bacillus* sp. CT04 and *Enterobacter* sp. (*Enterobacter* sp. DD01), killing potential of *Bacillus* sp. CT04 strain with the lowest LC_{50} and LT_{50} values among the four *Bacillus* and *Enterobacter* strains was realized. A satisfactory result with a fair reduction in leaf roller population was obtained after field application of the isolates. In case of *Eterusia magnifica* the most pathogenic strain was found to be *Bacillus* sp. RS01.

To be effective, it is necessary to bear in mind that several factors can affect the performance of the microbial pesticides. First the time of application is crucial since to some insect pests, only the first and second larval instars are susceptible. To achieve the best application time, a good sampling program is strongly recommended that involves the larval stage (susceptible window) and concentration of the microbial pesticide (Guerrero *et al*, 2007). Steinhaus (1951) produced in mass a spore-crystal preparation on agar in large Roux bottles for a field experiment and he successfully controlled the alfalfa caterpillars in field (Burges, 2001). The application dose of microbial pesticide is important as it should ensure the correct covering of the plant organs which are attacked by insect pest (Mocioni and Gullino, 2006; Guerrero *et al*, 2007). The biopesticide in liquid/water formulation is advantageous as it can be sprayed cost effectively at a high potency using ultra low volume appliances (Burges, 2001).

A correct management of these variables will ensure a greater efficacy in bio-pesticide application and will permit to reduce the number of pest attack, with obvious economic and environmental advantages. Further, field studies of the isolated strains of entomopathogenic bacteria are necessary to determine their exact potential to kill their hosts (=pests) in natural environmental condition where weather parameters play a major role.

7 References

7. References

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8 Summary

Summary

- Tea, the wonder beverage was first named *Thea sinensis* and is now known as *Camellia sinensis*. All over the world 'tea' is a popular invigorating and refreshing drink having excellent medicinal properties. It is the main agro-industry of North-East India including the Dooars, Terai and the Darjeeling foothill region.

- Different problems are associated with tea crop production, among which one of the major problems is crop loss due to pest attack, specially insect pests.

- About 300 species of arthropod pests are known to attack tea in India, lepidoptera form the largest order of the pest species (Chen and Chen, 1989).The chewing caterpillars generally may cause up to 40% crop loss.

- Among the lepidopteran tea pests, looper (*Buzura suppressaria)* caterpillar is one of the major defoliating pests of tea. Besides this, a new species of looper has emerged in the Dooars and Terai region, called black inch worm *Hyposidra talaca*. It is also causing substantial loss of tea crop.

- Red slug (*Eterusia magnifica*) caterpillar is one of the major defoliating tea pests of North-East India. The pest attacks mature tea leaves and becomes abundant in pockets of Terai-Dooars plantations.

- The tea leaf roller *Caloptilia theivora* has been known to occur in North-East India since 1988, infesting about 40-60% of the shoots in young and mature tea. It exploits young tea leaves while nesting inside a rolled-up leaf and deposits its faeces in the nest. The faecal contaminations of the made tea causes deterioration of quality, hence lower price of the product.

- To overcome the crop loss, population of tea pests are mostly managed chemically by synthetic insecticides. Various problems are associated with excess use of chemical pesticides. Besides affecting tea export due to high pesticide residue (MRL), these also affect human health, non-targets, environment and induce resistance in the target pests.

- In view of these problems associated with synthetic pesticides, and also due to a greater acceptance of organic tea (as compared to 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 in the tea ecosystem.

- The pesticide formulations in which entomopathogenic bacteria are the active components are known as bacterial pesticides. Numerous subspecies and strains of *Bacillus thuringiensis* have been isolated from dead and dying insect larvae, and in most cases the isolate showed toxic activity to the insect from which it had been isolated.

- In the present research study, a survey of the naturally occurring bacterial bio-agents occurring in the lepidopteran pests of tea from Terai and the Dooars was done. Entomopathogenic bacteria that were naturally infecting and killing the major lepidopteran pests were collected, isolated, characterized, then bioassayed in laboratory and field. Testing was done to determine their bio-efficacy, so that in future, microbial pesticides may be developed out of these, and the same may be integrated in biocontrol and IPM programs of tea.

- For knowing the natural occurrence of the entomopathogenic bacteria, population sampling of the host insects (*B. suppressaria*, *H. talaca*, *C. theivora* and *E. magnifica*) was done randomly from the tea plantations of Terai and the Dooars of Darjeeling foothill region and the adjoining plains.

- Dead and diseased larvae were collected from the field and from laboratory reared population and were surface sterilized with 70% alcohol and stored in double distilled water in refrigerator at –20°C, for bacterial isolation in future.

- Koch's postulate with the bacterial isolate was tested and the bacteria were characterized following morphological, biochemical and physiological procedures.

- After characterization, the bacteria were bioassayed with determination of the percentage mortality, LC_{50} and LT_{50} values in laboratory.

- Bioassay of cross-infectivity to lepidopteran tea pests of same habitat was performed.

- The most commonly occurring entomopathogenic bacterial strains of loopers were: *Bacillus* sp. BS01 of *B. suppressaria*, *Bacillus* sp. HT01 and *Bacillus* sp. HT02 of *H. talaca*. Several other bacterial strains (fourteen) isolated from the loopers were occurring in less frequency.

- Cross-infectivity to beneficial lepidopteran like silk worm was also determined before conducting field experiment.

- Field killing efficacy of the bacterial isolate (HT01) was determined by spraying in aqueous medium in RBD at Terai tea plantations.

- The three *Bacillus* strains (BS01, HT01, HT02) were found to be different from each other and also from commercial formulation of *Bacillus thuringiensis kurstaki* in respect of characteristics and infectivity. The LC_{50} and LT_{50} values were found to be lower in case of *Bacillus* sp. HT01 than the other two bacteria and from *Btk*. A field application of this bacterial strain (HT01) was conducted and a significant control of *H. talaca* population was obtained within 7 days at all concentration (without any sticker or spreader).

- In case of *Caloptilia theivora* four *Bacillus* strains (*Bacillus* sp. CT01, *Bacillus* sp. CT02, *Bacillus* sp. CT03 and *Bacillus* sp. CT04), and a strain of *Enterobacter* (*Enterobacter* sp. DD01) were isolated.

- The bioassay revealed that the bioassay it was found that the LC_{50} and LT_{50} values were lower in case of *Bacillus* sp. CT04 as compared to other four *Bacillus* strains and *Btk*. A field study was therefore conducted to know the field efficacy of the *Bacillus* sp. CT04 and a successful control was obtained on 7^{th} day at 3000 and 4000µg/ml concentrations.

- A bacterial strain named *Bacillus* sp. RS01 was isolated from *Eterusia magnifica*. The strain was found to be fairly pathogenic for killing early stage caterpillars of *E. magnifica*. Bioassay and field study conducted with *Bacillus* sp. RS01 strain to know its exact toxicity under field conditions registered a successful control of *E. magnifica* population but at a high concentration of 12,000µg/ml within 7 days.

- Report of development of insect resistance to *Btk* has stimulated new research to find additional *Bt* strains that have specific activity spectrum against the concerned insect pests. So, after considering all the findings it may be said that the bacterial strains (*Bacillus* sp. BS01; HT01, HT02; CT01, CT02, CT03, CT04; RS01 and *Enterobacter* sp. DD01) may be taken as potential candidates and developed in future as microbial biopesticides with a better killing efficacy than the commercially available *Bacillus thuringiensis kurstaki* (*Btk*) which is already in used for controlling different lepidopteran tea pests.

9 Highlights

Highlights of the findings

- Tea, *Camellia sinensis* is the main agro-industry of North-East India including the Dooars, Terai and Darjeeling foothill region.

- Insects specially lepidopteran tea pests such as loopers (*Buzura suppressaria* and *Hyposidra talaca*) cause major defoliations of young leaves, where as red slug caterpillars (*Eterusia magnifica*) mainly attack the mature tea leaves. Very young tea leaves are rolled-up as nest by the tiny caterpillars of *Caloptilia theivora*.

- Entomopathogenic bacteria that naturally infect and kill the pests were surveyed, isolated from caterpillar pests, characterized, then bioassayed and field tested to determine their killing efficacy.

- For knowing the natural occurrence of the entomopathogenic bacteria, population sampling of the host (pest) insects (*B. suppressaria*, *H. talaca*, *C. theivora* and *E. magnifica*) was done randomly from the tea plantations of Darjeeling foothill region and the adjoining plains (Terai and the Dooars).

- High rate of bacterial infection was recorded in all the concerned pests during the monsoon months.

- After satisfying Koch's postulate test, the bacteria were characterized following morphological, biochemical and physiological procedures.

- Characterization revealed presence of three bacterial strains, *Bacillus* sp. BS01 from *B. suppressaria* and *Bacillus* sp. HT01 and *Bacillus* sp. HT02 from *H. talaca* population.

- In case of *Caloptilia theivora* four *Bacillus* strains (*Bacillus* sp. CT01, *Bacillus* sp. CT02, *Bacillus* sp. CT03 and *Bacillus* sp. CT04) were isolated. Besides this, a non-spore forming strain of *Enterobacter* (*Enterobacter* sp. DD01) was isolated.

- From *Eterusia magnifica* caterpillars, a bacterial strain named *Bacillus* sp. RS01 was isolated.

- Cross-infectivity to other looper species was registered for *Bacillus* sp. BS01, HT01 and HT02. But none of the bacterial isolates were infective to *Bombyx mori nistari.*

- The isolated bacterial strains (*Bacillus* sp. BS01, HT01, HT02, CT01, CT02, CT03, CT04, RS01 and *Enterobacter* sp. DD01) from the tea lepidopterans showed good killing efficacy, based on laboratory bioassay.

- The findings reveal the spectrum of diversity of the bacterial entomopathogens of lepidopteran pests of tea for the first time.

- Field assay of only the most pathogenic strains, having low LC_{50} values, were tried.

- *Bacillus* sp. HT01 proved effective in controlling looper populations (*Hyposidra talaca*) within seven days of spray.

- *Bacillus* sp. CT04 was effective in significantly reducing the leaf roller population (*Caloptilia theivora*) within seven days at 4000µg/ml and 3000µg/ml concentrations.

- *Bacillus* sp. RS01 in field at high concentrations i.e., 12,000µg/ml and 11,000µg/ml proved effective in reducing red slug caterpillar population (*Eterusia magnifica*) within 7 days

- It is recommended that the field applied strains of *Bacillus* (HT01, CT04 and RS01) that have proved their killing potential against their concerned pests may be taken up for further formation, toxicity testing and development as commercial biopesticides.

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Date: 05.02.2010

Place: Siliguri

Damayanti De

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Annexure

Annexures

Annexure I

Abbreviation	Full name
1. LC_{50}	1. Lethal Concentration 50
2. LT_{50}	2. Lethal Time 50
3. ANOVA	3. Analysis of Variance
4. Conc.	4. Concentration
5. %	5. Percentage
6. O.D.	6. Optical Density
7. sp.	7. Species
8. Fig.	8. Figure
9. °C	9. Degree centigrade
10. rpm	10. Revolution per minute
11. Min	11. Minute
12. nm	12. Nanometer
13. DTT	13. Dithiothreitol
14. SDS-PAGE	14. Sodium dodecylsulphate polyacrylamide gel electrophoresis
15. LB	15. Luria Bertani
16. h	16. Hour
17. TV	17. Tea variety
18. ml	18. Milliliter
19. DFLs	19. Disease free layings
20. RBD	20. Randomized block design
21. T.E.	21. Tea Estate
22. SSP	22. Smith Statistical Package
23. kDa	23. Kilo Dalton
24. $\chi 2$	24. Chi Square

Annexure II

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Mukhopadhyay, A. and De, D. 2009. Pathogenecity of a baculovirus isolated from *Arctornis Submarginata* (Walker) (Lepidoptera:Lymantriidae), a potential pest of tea growing in the Darjeeling foothills of India. ***Journal of Invertebrate Pathology*. 100:** 54-56.

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Reprints

Research Article

Journal of Plantation Crops, 2008, 36 (3): 408-413

Pathogenicity of two strains of *Bacillus* infecting the lepidopteran tea pests, *Buzura suppressaria* and *Eterusia magnifica* in Darjeeling foothill region

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Abstract

Bacillus sp. BS01 and *Bacillus* sp. RS01 were isolated from diseased caterpillars of leaf feeding pests of tea, *Buzura suppressaria* and *Eterusia magnifica*, respectively from the Darjeeling foothill region. They were studied for their morphological, biochemical and physiological parameters and compared with the *Bacillus thuringiensis kurstaki* (*Btk*) strain used as commercial microbial pesticide. Analysis based on polyphasic approach such as growth phase, biochemical tests, whole body protein, crystal protein profiles along with bioassay (i.e. LC_{50} and LT_{50} values) of the bacterial isolates established these as two different strains of *Bacillus* sp. They differed in biochemical testing, growth phase, whole body protein profile, crystal protein profile, trypsinization of crystal protein and LC_{50} and LT_{50} values. These two strains were also distinct from commercial microbial pesticide *Btk*. The biochemical characteristics in particular the whole body and crystal protein profiles of the two *Bacillus* strains showed marked difference from those of *Btk*. The generation time of *Btk* was lower than either of the two isolated *Bacillus* strains. These new strains isolated from the host insects open up the possibility of their future use as microbial pesticide after further development in their formulation and determining their safety standards.

Keywords: Buzura suppressaria, Bacillus sp. BS01, *Bacillus* sp. RS01, *Camellia sinensis*, Darjeeling, *Eterusia magnifica*, Tea

Introduction

Tea is the main agro-industry of Darjeeling hills and its adjoining plains of the Dooars, Terai and the North-East region of India. But the plantation of this region is severely damaged by attacks of lepidopteran tea pests. *Buzura suppressaria* commonly called as looper and *Eterusia magnifica* called as red slug caterpillar cause a substantial loss by defoliating the crop both quality and quantity of tea. To control the attack of these tea pests, the present practice is largely based on the use of synthetic pesticides, with some back lashes such as environmental pollution, human health hazard, resistance in pests (Sarker and Mukhopadhyay, 2006) and MRL problems affecting export of tea. 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 production of export quality tea through organic farming.

Strains of *Bacillus* sp. BS01 and *Bacillus* sp. RS01 were isolated from two of the lepidopteran pests of tea that showed natural diseased condition in plantation. Growth rate, biochemical testing, vegetative protein profile, crystal protein profile, trypsinization of crystal and bioassay i.e. LC_{50} and LT_{50} values of the two strains proved them to be similar to *Bacillus thuringiensis* but differing in their strains or subspecies characters. They also differed from *Bacillus thuringiensis kurstaki*, which is commonly sprayed in garden with marginal control of these pests.

The present study was aimed to isolate and characterize the naturally occurring bacterial pathogens of *B. suppressaria* and *E. magnifica* and to know their potential as biopesticide through determination of their median lethal concentration and time. *Bacillus thuringiensis kurstaki* was used as a reference for comparison.

Materials and Methods

Bacterial strains were isolated from larvae of *B. suppressaria* and *E. magnifica*. For isolation of bacteria, the larvae dying of disease were taken for surface sterilization with 70% alcohol and then washed three

times with sterilized double distilled water. Then they were stored in double distilled water within sterilized eppendorf tube. The eppendorf 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 and the 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 were determined following Koch's postulates by infecting healthy first instar larvae with these two bacteria. After proving the Koch's postulates the viability was checked by inoculating in newly prepared agar medium at weekly intervals.

Characterization of the strain BS01 and RS01

Morphological characteristics

Cell, spore shape and structure of crystal protein, colony texture, motility observed for the two bacterial isolates and the same were compared with *Bacillus thuringiensis kurstaki* used as reference.

Biochemical characteristics

Several biochemical tests 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 Biochemcial testing kit (KB003) (Himedia) with *Btk* as reference.

Physiological characteristics

Doubling or Generation time (Cappuccino and Sherman, 1996)

Growth of the bacterium was determined by turbidimetric method. The O.D. value was taken at 540 nm in spectrophotometer at every 30 minutes interval. Finally optical density and time interval were plotted on a graph paper and from this the generation time (doubling time) was determined. *Btk* was taken as reference.

SDS-PAGE profile of crystal protein

Bacterial strains were grown in Luria Bertani medium at 37°C without shaking. It was grown up to the phase of sporulation. The crystal was harvested in high pH buffer of sodium carbonate and 2-marcaptoethanol after Kranthi (2005) with slight modification [2-Marcaptoethanol was used instead of Dithiothreitol (DTT)]. This crystal protein was taken for SDS-PAGE analysis.

Trypsinization of crystal proteins

Aliquotes (100µl) of solubilized crystal proteins of the bacterial strains were subjected to trypsin treatment at 37°C in a 10:1 (vol/vol) soluble protein/trypsin ratio with 1 mg of trypsin per ml dissolved in sterilized double distilled water. After two hours incubation, samples were analyzed by SDS-PAGE (Lopez-Meza, 1996).

SDS-PAGE analysis of vegetative protein of the bacterium

For SDS-PAGE analysis of vegetative protein of Gram positive bacteria, 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, pH 6.8, and 4% deionized water and analyzed by SDS-PAGE according to the method described by Costas (1992). Gels were fixed in trichloroacetic acid and were stained with Brilliant blue G-250 (Neuhoff *et al.*, 1988; Konecka *et al.*, 2006).

Bioassay

Crude spore crystal mixture (100, 300, 500, 750 and 1000 µg/ml) of the bacteria isolated from *B. suppressaria* and *E. magnifica* respectively were used for bioassay after the method of Unnamalai and Sekar, (1995). Leaves dipped in different concentrations were offered as food. Second instar larvae (n=100) were used for each treatment (concentration). Sterile distilled water was used in control. The mortality was observed at an interval of 24 h from the day of inoculation (bioassay testing). The corrected mortality was calculated using Abbott's formula. Data were subjected to probit analysis after Finney (1954), and median lethal concentration (LC_{50}) value was calculated from the regression equation. Value of median lethal time (LT_{50}) was also determined simultaneously following the method of Biever and Hostetter (1971).

Results and Discussion

Characterization of the two bacterial isolates (BS01 and RS01)

All the morphological characteristics like vegetative body structure, spore-shape, motility, colony texture, crystal protein shape of the isolated bacterial strains BS01 from *B. suppressaria* and RS01 from *E. magnifica* were found to be similar with that of *Bacillus thuringiensis kurstaki*. BS01 and RS01 showed all the characteristics of genus *Bacillus* which included rod shaped vegetative body, endospore formation, gram

positivity, facultative anaerobic nature, catalase positivity, acid production from glucose and motility (Sneath, 1986).

Characteristics of the two *Bacillus* strains (BS01 and RS01) showed same biochemical properties except in nitrate reduction test where BS01 showed negative reaction and RS01 showed positive reaction. However, these two strains showed much difference in their biochemical tests, when compared with the results of *Btk* (Table 1).

Table 1. Comparative account of biochemical tests of BS01, RS01 and *Btk*

Biochemical Characteristics	*Btk*	BS01	RS01
ONPG	+	-	-
Lysine decarboxylase	+	+	+
Ornithin decarboxylase	+	+	+
Urease	-	+	+
Phenyl alanine 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	-	+	+
Sorbitol	-	-	-

Growth phase or determination of doubling time

The generation or doubling time of the two strains was much different. The doubling time was 60 min in case of BS01 and 120 min in case of RS01. The *Btk* which was used as reference had totally different doubling time from the above two strains.

SDS-PAGE profile of crystal protein (trypsin untreated)

When protein composition was analyzed by SDS-PAGE, crystals of BS01 showed difference in banding pattern with RS01. Bands of molecular weight 53 and 49 kDa were present in RS01 strain but were absent in BS01 strain. In BS01 strain a 52 kDa molecular weight protein band was present which was absent in RS01 strain. The BS01 strain differed from *Btk* in 42 kDa position whereas RS01 differed in 52 and 42 kDa banding position. So, a major difference in banding pattern was found between BS01 and RS01 and *Btk* (Fig. 1).

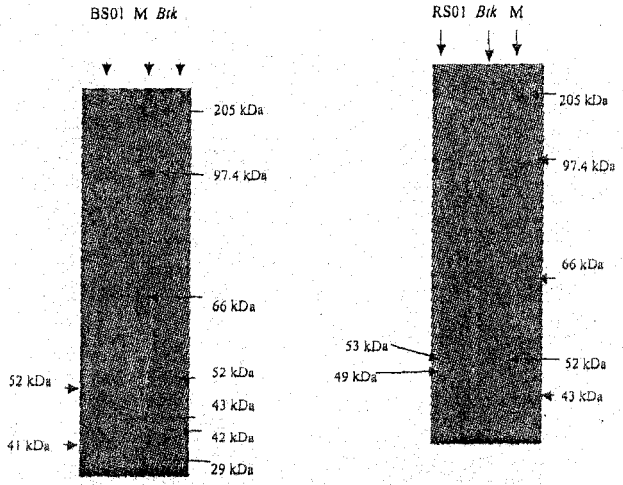

Fig. 1. SDS-PAGE profile of crystal protein of BS01, RS01 and *Btk*

Trypsinization of crystal protein

The presence of any putative peptide fragment resistant to tryptic digestion in the BS01 and RS01 strains crystals were verified. After solubilization the crystals were treated with trypsin. Trypsin digestion revealed the trypsin resistant peptides. The expected molecular masses of which on SDS-PAGE gel were 49, 44 and 38 kDa in case of BS01 and in case of RS01 strain these were 51 and 43 kDa molecular weight peptides (Fig. 2). Whereas in case of *Btk* it was 49, 44 and 40 kDa. So differences in banding pattern were found between BS01 and RS01 which also differed from *Btk*. This experimental result revealed that 49, 44 and 38 kDa molecular weight peptides were the toxic components of BS01 and 51 and 38 kDa of RS01 strains, respectively.

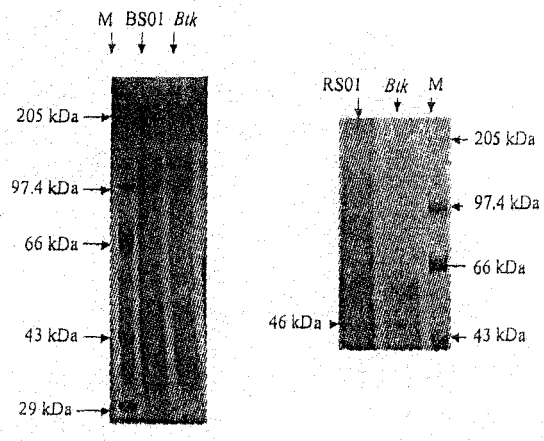

Fig. 2. Trypsinization of crystal protein of BS01, RS01 and *Btk*

Whole cell protein profile

Difference in banding pattern of whole body protein profile was found between *Btk* and BS01 strains. Three major protein bands (43, 34 and 30 kDa) were present in BS01 strain which were absent in *Btk*. On the other hand two major protein bands (44 and 31 kDa) were present in *Btk* which were absent in BS01 strain. In case of RS01 strain, 44 kDa protein band was present which was totally absent in BS01 strain but present in *Btk*. So, major differences at cellular protein level present between BS01, RS01 and *Btk* strains (Fig. 3).

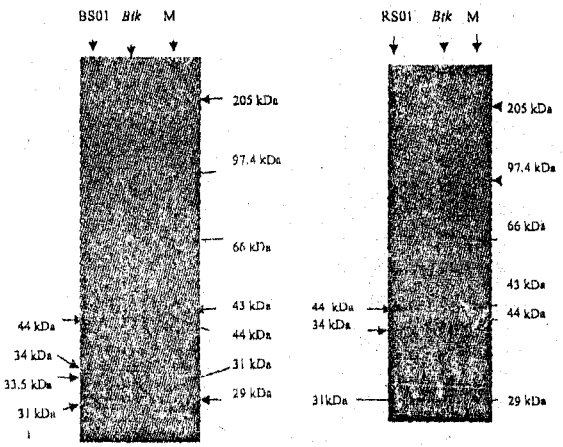

Fig. 3. SDS-PAGE profile of vegetative protein of BS01, RS01 and *Btk*

Bioassay

The mortality of second instar *B. suppressaria* caterpillars varied from 81 to 3% within 9 days. The LC_{50} value was found to be 446.7 µg/ml with fiducial limits 407.96 µg/ml (lower limit) 485.44 µg/ml (upper limit). The LT_{50} values were found to be 6.19 days for 1000 µg/ml, 6.5 days for 750 µg/ml and 8.92 days for 500 µg/ml concentrations. In case of RS01 strain the LC_{50} value was found to be 458.2 µg/ml with fiducial limits 457.955 µg/ml (lower limit) and 458.245 µg/ml (upper limit). The LT_{50} values were found to be 5.6 days for 1000µg/ml, 5.69 days for 750µg/ml and 6.19 days for 500µg/ml concentrations. On the other hand in case of *Btk* which was used against *B. suppressaria* the LC_{50} value was found to be 524.8µg/ml with 462.655µg/ml (lower limit) 586.945µg/ml (upper limit). The LT_{50} values were found to be 6.35 days for 1000µg/ml and 8.91 days for 750µg/ml. The LC_{50} value of *Btk* which was used against *E. magnifica* was found to be 416.9µg/ml with 416.76µg/ml (lower limit) and 417.034µg/ml (upper limit). The LT_{50} values were found to be 6.67 days for 1000µg/ml, 6.93 days for 750µg/ml and 7.54 days for 500 µg/ml concentrations (Table 2).

The two *Bacillus* strains BS01 and RS01 isolated from *B. suppressaria* and *E. magnifica* showed typical characteristics of *Bacillus thuringiensis* especially in their vegetative body structure and crystal production during sporulation. As crystals are the typical distinguishing characteristics of *Bt* (Heimple and Angus, 1958; Bai *et al.*, 2002), the isolates were identified as *Bt* strains on the basis of the crystal formation (Bai *et al.*, 2002; Brussock and Currier, 1990). However, on biochemical testing of ONPG, Lysine decarboxylase, Ornithin decarboxylase, Urease, Nitrate reduction, V-P, acid production from glucose etc. they showed difference among themselves (BS01 and RS01) and also with *Bacillus thuringiensis kurstaki*, the latter being used as microbial pesticide against lepidopteran pests. The growth pattern that is the generation time was found to be different among the two isolates and also with the commercial formulation of *Btk*. The crystal protein profile was found to be different amongst two isolates also *Btk*. Further the trypsinized crystal proteins also differed among the strains. The vegetative protein profile in SDS-PAGE was found to be decisively different among the two strains and also from *Btk*.

In case of bioassay, these two strains were found to be highly toxic to their respective hosts from which they had been isolated. Due to their close median lethal concentration values (LC_{50}) which were not much different from the commercially used *Btk*, developing these new strains into commercial pesticides, for controlling of *B. suppressaria* and *E. magnifica* caterpillars offers great potential. The LT_{50} values of these strains were also comparatively lower than the commercially used bacterium, *Btk*. The possibility of finding cross infectivity of these strains to other harmful lepidopteran pests is still open and bioassay against other insect pests is under way.

Report of development of insect resistance to *Btk* has stimulated new research to find additional *Bt* strains and other microbes that have specific spectrum against certain insects (Bai *et al.*, 2002; Mc. Gaughey, 1985; Monnerat *et al.*, 2000; Salama and Abdel-Razek, 2000).

Bacillus sp. was reported earlier (De *et al.*, 2006) from *B. suppressaria* and a detail account has already been furnished in this report. But as no *Bacillus* strain has so far been reported from *E. magnifica* of sub Himalayan tea plantation, the isolated strain (*Bacillus* sp. RS01) is a new report. Based on the distinguishing

Table 2. Comparative account of LC_{50} and LT_{50} values of BS01, RS01 and *Btk*.

Name of bacterium	Mortality (%)	LC_{50} with Fiducial limits	LT_{50}	Hetero-geneity	Regression
Btk tested on *B. suppressaria*	75%for 1000 µg/ml 60%for 750 µg/ml 48%for 500 µg/ml 18%for 300 µg/ml 17%for 100 µg/ml	524.8µg/ml With 462.655µg/ml (Lower limit) 586.945µg/ml (Upper limit)	6.35 for 1000µg/ml 8.91 for 750µg/ml	$\chi^2(5)$=120 for 1000µg/ml $\chi^2(5)$=85.7143 for for 750 µg/ml $\chi^2(5)$=63.1579 for 500 µg/ml $\chi^2(5)$=19.7802 for 300 µg/ml $\chi^2(5)$=18.5792 for100µg/ml	Y=1.669X+2.253
BS01 tested on *B. suppressaria*	81% for 1000 µg/ml 59% for 750 µg/ml54 % for 500 µg/ml 6% for 300 µg/ml 3% for 100 µg/ml	446.7 µg/ml With 407.96 µg/ml (Lower limit) 485.44 µg/ml (Upper limit)	6.19 for 1000µg/ml 6.5 for 750µg/ml 8.92 for 500µg/ml	$\chi^2(5)$=3.0457 for 100 µg/ml $\chi^2(5)$=6.1856 for 300 µg/ml $\chi^2(5)$=73.9726 for 500 µg/ml $\chi^2(5)$=83.6879 for 750 µg/ml $\chi^2(5)$=136.1345 for 1000 µg/ml	Y=3.33X-21.3
Btk tested on *E. magnifica*	87% for 1000 µg/ml 83%for 750 µg/ml 77%for 500 µg/ml 21%for 300 µg/ml 20%for 100 µg/ml	416.9µg/ml With 416.76µg/ml (Lower limit) 417.034µg/ml (Upper limit)	6.67 for 1000µg/ml 6.93 for 750µg/ml 7.54 for 500 µg/ml	$\chi^2(5)$=118.6868 for 1000 µg/ml $\chi^2(5)$=107.1048 for 750 µg/ml $\chi^2(5)$=91.3234 for 500 µg/ml $\chi^2(5)$=4.6192 for 300 µg/ml $\chi^2(5)$=3.9216 for 100 µg/ml	Y=2.61X-8.81
RS01 tested on *E. magnifica*	78% for 1000 µg/ml 72% for 750 µg/ml 61 % for 500 µg/ml 21% for 300 µg/ml 20% for 100 µg/ml	458.2 µg/ml With 457.955 µg/ml (Lower limit) 458.245 µg/ml (Upper limit)	5.6 for 1000µg/ml 5.69 for 750µg/ml 6.19 for 500µg/ml	$\chi^2(5)$= 93.8312 for 1000 µg/ml $\chi^2(5)$=79.4543 for 750 µg/ml $\chi^2(5)$=56.7966 for 500 µg/ml $\chi^2(5)$=4.6192 for 300 µg/ml $\chi^2(5)$=3.9216 for 100 µg/ml	Y=2.16X-4.16

characters and the killing efficacy, the *Bacillus* sp. BS01 and *Bacillus* sp. RS01 hold a promise to be developed in future as microbial pesticides.

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Corrigendum:

Trypsinization of crystal protein:

The presence of any putative peptide fragment resistant to tryptic digestion in the BS01 and RS01 strains crystals were verified. After solubilization the crystals were treated with trypsin. Trypsin digestion revealed the trypsin resistant peptides. It was found that no difference in banding pattern was there between BS01 and *Btk*. But a 46 kDa molecular weight protein band was there in RS01 strain which was totally absent in *Btk* (Fig. 2). So difference in banding pattern were found between BS01 and RS01 which also differed from *Btk*

Whole cell protein profile:

Difference in banding pattern of whole body protein profile was found between *Btk* and BS01 strains. Two major protein bands (44 and 31 kDa) were present both in *Btk* and BS01 strain. But the difference was found in 33.5 and 34 kDa molecular weight protein bands which were present only in BS01 strain but absent in *Btk*. On the other hand one protein band of 31 kDa present in *Btk*, was absent in RS01 strain. Instead in RS01 strain a 34 kDa protein band was present which was totally absent in *Btk*. So, major differences were evident between RS01 and *Btk* strain (Fig. 3).

Short Scientific Report

Journal of Plantation Crops, 2007, 35 (2) : 122-124

A report on the naturally occurr ng pathogenic bacteria of the lepidopteran tea pest, *Buzura suppressaria* (Lepidoptera: Geometridae), from Darje(ling foothills and plains

(Manuscript Received: 27-02-06; Revise : 17-05-06, Accepted: 26-04-07)

Keywords: Buzura suppressaria, Camellia sinensis, bacterial pa ıogen, Darjeeling

The looper caterpillar, *Buzura suppressaria* Guenee (Le ıidoptera: Geometridae), attacks and defoliates mat ıre tea, *Camellia sinensis* (L.) O. Kuntze causing heavy crop loss (up to 40%) (Banerjee, 1993; Anonymous, 1994). A bacterial pathogen caused mo tality of the populations of 2nd and the 3rd brood of *B. suppressaria* (Das, 1965). In recent years, the looper act vities have greatly increased due to its greater tole rance to chemical pesticides of organophosphate and syn thetic pyrethroids (Sannigrahi and Talukdar, 2003; Sarker and Mukhopadhyay, 2006). In view of this, efforts are being made to evolve alternate strategies for the management of this pest through application of microbial bio agents.

B. thuringiensis formulations have been applied eff ectively against tea pests, such as *Caloptilia theivora* an l *Cydia leucostoma* (Unnamalai and Sekar, 1995) and *B. suppressaria* (Das and Barua, 1990). It has also being su ccessfully used in controlling the tea totricid ca erpillars (Kariya, 1977) and other lepidopteran pests (M uraleedharan and Selvasundaram, 2002).

The present study was aimed to isolate and ch aracterize the naturally occurring bacterial pathogen of *B. suppressaria* up to genus level and then conduct bi ıassay to determine the lethal concentration (LC_{50}) and le hal time (LT_{50}).

200-250 larvae of *B. suppressaria* were collected on T ıcklai vegetative clone, TV-25, from mature tea pl antations about 20 years old of Darjeeling foothills and it adjoining plains. These were reared in laboratory for tw o generations at 27 ± 2 °C; 72 ± 2 % RH with a p otoperiod (L: D) of 13:11 hrs. in aseptic conditions. Fresh tea twigs (TV-25) from the experimental tea garden maintained on North Bengal University campus was supplied as food. The two-day old second instar larvae (0.015 g) were used for bioassay test.

For isolation of bacteria, dead larvae were collected during February-March and putrefied for 5 days with autoclaved double distilled water in a sterile glass container. Whole body was thoroughly macerated by glass homogenizer and the 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 stored in autoclaved eppendorf tube with double distilled water at –20 °C (Lacey and Brooks, 1997). Pure culture of the bacterium was isolated from mixed populations by dilution streak method in nutrient agar medium. Viability was checked by inoculating in new agar medium at weekly intervals. To characterize the bacterium up to genus level some experiments were conducted. Gram staining followed by Catalase assay and Voges-Proskour (V-P) reaction were performed with the bacterial isolate after the method of Thiery and Frachon (1997). Subsequently its anaerobic growth was also observed (Sneath, 1986). To find out whether spores were present in the body of bacterium, a staining procedure was applied with 0.5% Basic fuchsin. Growth phase of the isolate (*Bacillus* sp.) and *Bacillus thuringiensis* kurstaki (from IARI, New Delhi) was compared and their doubling time was determined after Cappucino and Sherman (1996).

Extraction and bioassay of crude spore mixture was done after the procedure of Dulmage *et al.* (1970) with some modifications. Different concentrations of crude

samples (spores) (500, 750 and 1000 µg/ml) 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 Sekar (1995). Twenty second instar larvae per concentration @ three replicates were fed and sterile distilled water was used in control. The mortality was observed at an interval of 24 hrs from the day of inoculation (bioassay testing). The corrected mortality was calculated using Abbott's formula (Abbott, 1925). Data were subjected to probit analysis (Finney, 1954) 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):

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

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.

The larvae of infected *B. suppressaria* turned brownish with putrification and liquefactions of tissue. The bacterium isolated from such cadavers showed characteristic colony formation, growth pattern, and sporulation. Further, the catalase assay, V-P reaction and anaerobic growth results favoured the identity of the bacterium as one close to *Bacillus thuringiensis*. However, the bacterium differed from the commonly occurring lepidopteran strain *B. thuringiensis* kurstaki by a faster growth rate (Fig. 1) and its pathogenicity (Per. Comm.). The doubling time of the newly identified *Bacillus* sp. was 60 min (approx.) whereas for *B. thuringiensis* kurstaki was about 90 min. Due to the bacterial infection the mortality of *B. suppressaria* in the natural population varied from 40-60% in different seasons i.e. during pre-monsoon (March- June), monsoon (July- October) and post-monsoon (November- February) (Fig. 2) with an average mortality of about 50%. It is reported that bacterial disease takes a heavy toll of 2nd and 3rd broods of *B. suppressaria* between May and August (Das, 1965). A formulation of isolate (*Bacillus* sp.) and its application in various doses with food proved the bacterial pathogen to be effective in causing dose dependant mortality of *B. suppressaria* caterpillars. Percentage mortality of *B. suppressaria*, when infected with different concentration of crude spore mixture of the isolate (*Bacillus* sp.), ranged from 41.66 to 80 in first 9 days (Table 1). The median lethal concentration (LC_{50}) was found to be 727.8 µg/ml and median lethal time (LT_{50}) was to be 8.05 days in case of 750 µg/ml spore concentration to 5.93 days in 1000 µg/ml spore concentration (Table 2). The lowest concentration of 500 µg/ml was not found effective in larvae as the caterpillars continued up to pupation.

Use of bacteria, especially *B. thuringiensis*, is well established as microbial pesticide for different tea pests (Kariya, 1977; Barbora, 1995). Efficacy study of different strains of *B. thuringiensis* along with determination of LC_{50} values has been done for tea leaf eating caterpillars, *Caloptilia theivora* (LC_{50} 0.296 µg/ml with max. conc. of crude sample 0.300 µg/cm²) and *Cydia leucostoma* (LC_{50} 0.221 µg/ml with max. conc. of crude sample 0.300 µg/cm²) (Unnamalai and Sekar, 1995). As proper identity and bioassay of the naturally occurring bacterium (*Bacillus* sp.) of *B. suppressaria* in question was so far not known, the present investigation substantiates to some

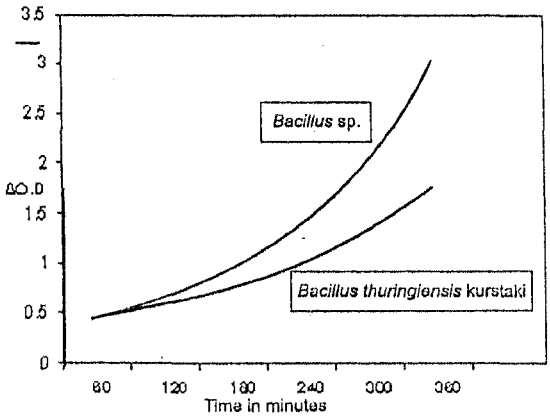

Fig. 1. Comparison of growth curves at exponential phase of the newly identified *Bacillus* sp. and *Bacillus thuringiensis* kurstaki.

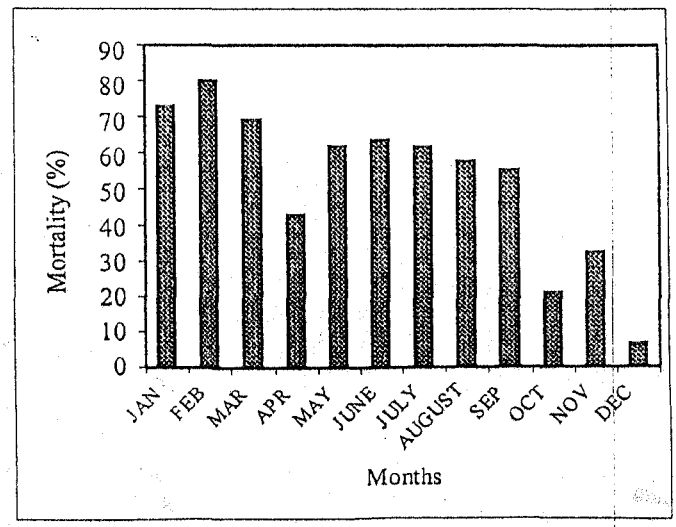

Fig. 2. Percentage mortality of *B. suppressaria* population collected from terai tea plantation in different months of the year

Table 1. Toxicity of *Bacillus* sp. against *Buzura suppressaria* within 2-9 days

Crude spore concentration of *Bacillus* sp. (µg/ml)	Log. Conc. (X)	No. of tested larvae	Actual mortality	Percentage mortality	Corrected mortality (%)
500	2.69	60	25	41.66	16.65
750	2.87	60	39	65	35.71
1000	3	60	48	80	71.42
Control	Nil	60	18	30	Nil

Heterogeneity	Regression	LC_{50}	Fiducial limits
$\chi 2(5)= 2415.92$	Y=8124.45-2836.75X	727.8	724.9 (Lower limit) 730.6 (Upper limit)

Table 2. Effect of different concentrations of spores (*Bacillus* sp.) on LT_{50} against second instar larvae of *B. suppressaria*

Dosage (µg/ml)	LT_{50} (days)	% Mortality
1000	5.93	80
750	8.05	65
500	43.36	41.66

extent its distinct nature as a *Bacillus* which is different from *Bacillus thuringiensis* kurstaki and its potential as a microbial bioagent.

Acknowledgement

The authors wish to thank Dr. R. Chakraborty, Head, Department of Biotechnology, University of North Bengal for extending the laboratory facility and Prof. P. K. Sarkar, Microbiology laboratory, Department of Botany, University of North Bengal for giving valuable suggestions. The authors are also thankful to Dr. Bishwajit Paul, Scientist (Senior scale), Entomology Division, IARI, New Delhi for his suggestion on the character of the bacterium and to an unknown reviewer.

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SHORT COMMUNICATION

A novel bacterial pathogen (*Enterobacter* sp.) isolated from the leaf roller, *Caloptilia theivora* of tea of Darjeeling foothills

Damayanti De · Ananda Mukhopadhyay · Ranadhir Chakraborty

Received: 5 January 2008 / Accepted: 17 April 2008 / Published online: 1 May 2008

Abstract *Enterobacter* sp. was isolated from the diseased and dead caterpillars of the tea leaf roller (*Caloptilia theivora*) from the Darjeeling foothill region. When the vegetative form of the bacterium was applied via food, mortality of *C. theivora* showed an LC_{50} value at 363.1 μg/ml (bacterial wt./vol. of water) with fiducial limits 363.25 and 362.94 μg/ml respectively. The LT_{50} values for *C. theivora* were 6 days for 100 μg/ml, 5.96 days for 300 μg/ml, 5.81 days for 500 μg/ml, 4.96 days for 750 μg/ml and 4.61 days for 1,000 μg/ml concentrations. The finding would enable one to contemplate development of a microbial pesticide using this novel *Enterobacter* sp. DD01 for control of the leaf rolling pest.

Keywords *Caloptilia theivora* · *Camellia sinensis* · *Enterobacter* sp. · Tea · Darjeeling

Introduction

The tea leaf-roller, *Caloptilia theivora* (Lepidoptera: Gracillariidae), attacks and consumes young leaves of tea bushes, *Camellia sinensis*, causing substantial loss in tea production and deterioration of its quality. Synthetic pesticide sprays have their limitation to readily reach caterpillars and pupae of the species that remain covered in the leaf rolls. Moreover, residue-free organic tea has a great demand in domestic and international (export) market. In view of this, biological control of this tea pest has become a necessity, which could possibly be achieved through development of microbial pesticides.

The present study was aimed to isolate and characterize the naturally killing bacterial pathogen of *C. theivora* followed by bioassay to determine lethal concentration (LC_{50}) and lethal time (LT_{50}) of *Enterobacter* sp. DD01 in order to find its potential.

Materials and methods

DD01 was isolated from larvae of *C. theivora*. For isolation of bacterium of *C. theivora*, the larvae dying of disease were taken for surface sterilization with 70% alcohol and then washed three times with sterilized double distilled water. Then they were kept in sterilized Eppendorf tube with sterilized double distilled water. The Eppendorf 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 and the crude homogenate was sieved through fine sterilized cotton cloth. The filtrate was centrifuged at 3,000 rev/min for 30 min. The supernatant was discarded and the precipitate containing bacterial suspension was taken for pure culture isolation by the 'dilution streak method' in nutrient agar medium. Two different types of colonies appeared on the nutrient agar medium after inoculation. One was the 'white glossy circular' colony and another was the 'yellow pigmented colony'.

From the white glossy circular colonies innocula were taken and dilution-streaked on four culture plates. The same was done in case of yellow pigmented colonies.

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From each of the four white glossy circular and yellow pigmented colonies, single colonies were purified on nutrient agar media. The purified strains having white glossy colony phenotype were named as WGC01, WGC02, WGC03 and WGC04 and similar yellow pigment-producing strains were named as YP01, YP02, YP03 and YP04. The purified cultures of all WGC and YP strains were individually fed to healthy *C. theivora* larvae. The result showed that only one white glossy circular-colony-producing strain (WGC03) created the disease syndrome in healthy larvae. On the other hand all four yellow pigmented culture failed to produce the disease syndrome. From the diseased larvae again plating on nutrient agar was repeated. The predominance of white glossy colonies was found on the culture plate. From WGC colonies single colony was purified which was named, DD01. From that DD01 stock, bacterial innoculum was taken to reinfect the healthy larvae for bioassay.

Several biochemical tests like indole, Voges-Proskauer, methyl red, citrate utilization, esculin hydrolysis lysine decarboxylase, ornithin decarboxylase, H_2S production, nitrate reduction, fermentation of different carbohydrates, urease tests were performed using Enterobacteriaceae Identification Kit (KB003 Hi25) (Himedia).

Aqueous formulation of the DD01 (vegetative body) (100, 300, 500, 750 and 1,000 μg/ml) of *C. theivora* was used for bioassay after the method of Jackson et al. 1992). Leaf dipped in different concentrations of the above bacterial solution were offered as food. Second instar larvae ($n = 100$) were used for each treatment (concentration). Treatment with sterile distilled water was used in the controls. The mortality was observed at an interval of 24 h from the day of inoculation (bioassay testing). The corrected mortality was calculated using Abbott's formula. Data were subjected to probit analysis (Finney 1954) and the median lethal concentration (LC_{50}) value was calculated from the regression equation. The median lethal time (LT_{50}) value was also determined simultaneously following the method of Biever and Hostetter (1971).

Results and discussions

Vegetative cells of the bacterial strain DD01 were aerobic, Gram-negative, motile and rod shaped. The strain was positive in Voges-Proskauer, β-galactosidase, lysine decarboxylase, ornithin decarboxylase, nitrate reduction, Simmons citrate, esculin hydrolysis tests, but negative in phenylalanine deaminase, H_2S production, methyl red, indole, oxidase, urea hydrolysis and gelatin liquefaction tests. It utilized malonate, L-arabinose, D-xylose, D-adonitol, L-rhamnose, cellobiose, mellibiose, saccharose, raffinose, trehalose, D-glucose, lactose and D-sorbitol. The bacterial strain, DD01 was therefore tentatively assigned to the genus *Enterobacter*. This *Enterobacter* sp. has thus been isolated for the first time from diseased caterpillars of *Caloptilia theivora* of tea grown in the Darjeeling foothill region. When the bacterium was applied via food, mortality of *C. theivora* showed the LC_{50} value at 363.1 μg/ml with fiducial limits 363.25 and 362.94 μg/ml, respectively. The LT_{50} values for *C. theivora* were 6 days for 100 μg/ml, 5.96 days for 300 μg/ml, 5.81 days for 500 μg/ml, 4.96 days for 750 μg/ml and 4.61 days for 1,000 μg/ml concentrations (Table 1).

The use of the Gram-negative non-spore forming bacterium *Serratia entomophila* is well established as microbial bioagents for controlling of grass grub larvae in New Zealand (Jackson et al. 1992). There are approximately 100 species of bacteria pathogenic to insects but

Table 1 LC_{50} and LT_{50} values of *Enterobacter* sp. DD01 for the pest *C. theivora* during first seven days of inoculation

Enterobacter sp. (μg/ml)	Log. Conc. (*X*)	No. of tested larvae (*n*)	Actual mortality	Percentage mortality (%)	LT_{50} (days)
100	2	100	64	64	6.0
300	2.47	100	74	74	5.96
500	2.69	100	76	76	5.81
750	2.87	100	79	79	4.96
1000	3	100	86	86	4.61
Control	0.0	100	20	20	–

Heterogeneity	Regression	LC_{50}	Fiducial limits
$\chi2(5) = 39.73$ for 100 μg/ml	$Y = 18.72 + 0.69X$	363.1 μg/ml	362.94 (Lower limit)
$\chi2(5) = 58.53$ for 300 μg/ml			363.25 (Upper limit)
$\chi2(5) = 62.82$ for 500 μg/ml			
$\chi2(5) = 69.62$ for 750 μg/ml			
$\chi2(5) = 87.43$ for 1,000 μg/ml			

hardly one percent of these are used as biocontrol agents. Nevertheless, the gut-colonizing bacterium *Enterobacter cloacae* has been reported to be used in pest control (Watanabe et al. 2000).

So, the present investigation reasonably substantiates the potentiality of the *Enterobacter* sp. DD01 bacterium as a microbial bioagent effective against the pest, *C. theivora*.

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CORRIGENDUM

Clarification of the points raised by the examiner of the Ph.D thesis of Smt. Damayanti De, Department of Zoology, NBU

(No: Ph.D./Zoo/910/R.11 dt: 20.5.2011)

Clarifications

Point no 1:

The standard style of presenting LC_{50} & LT_{50} tables has not been followed. Graphs showing dose response curve are lacking. The heterogeneity as shown in LC_{50} & LT_{50} tables should be checked.

LC_{50} & LT_{50} value have been furnished in tabular form where the column of mortality (response) has been presented against the dose/concentration of the entomopathogenic bacterial isolate. The results of 'dose-response' are further being presented graphically (Ref: Öztürk *et al.*, 2008; Nun˜ez-Valdez *et al.*, 2008) below as requested by the reviewer. The heterogeneity of LC_{50} is also checked as proposed.

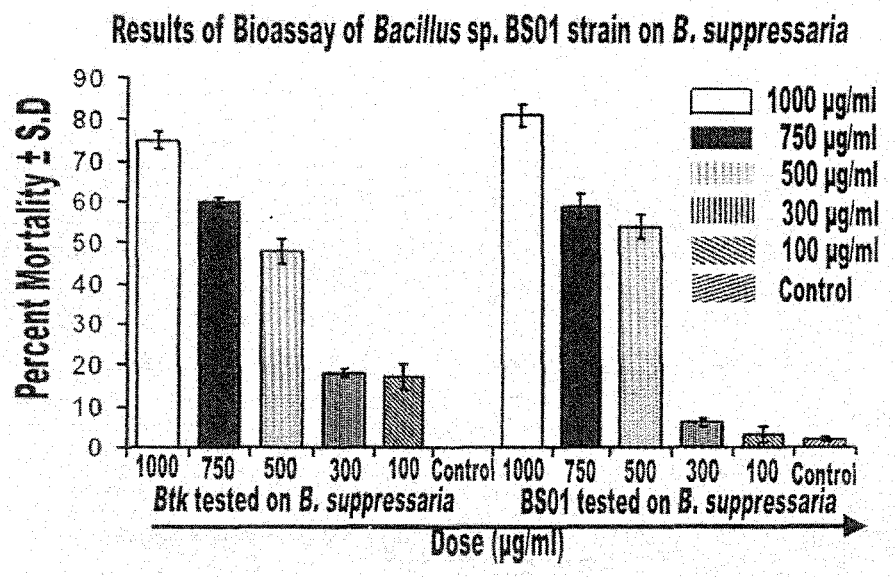
References:

Öztürk F., Açık L., Ayvaz A., Bozdoğan B and Suludere Z. 2008. Isolation and Characterization of Native *Bacillus thuringiensis* Strains from Soil and Testing the Bioactivity of Isolates Against *Ephestia Kuehniella* Zeller (Lepidoptera: Pyralidae) Larvae. *Turk J Biochem* 33(4):202-208.

Eugenia Nun˜ez-Valdez M., Calderón M A., Aranda E., Herna´ndez L., Ramı´rez-Gama R M., Lina L., Rodrı´guez-Segura Z., Gutie´rrez M C. and F J. 2008. Identification of a Putative Mexican Strain of *Serratia entomophila* Pathogenic against Root-Damaging Larvae of Scarabaeidae (Coleoptera). *Applied and Environmental Microbiology*. 74(3): 802-810.

Dose response curves are graphically presented below:

Graphical representation of table presented in page no. 51



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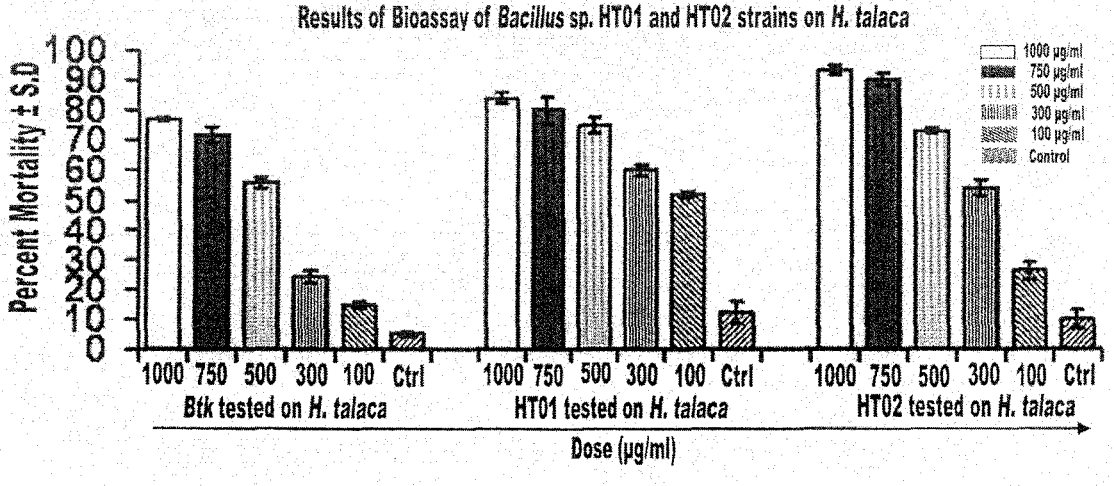

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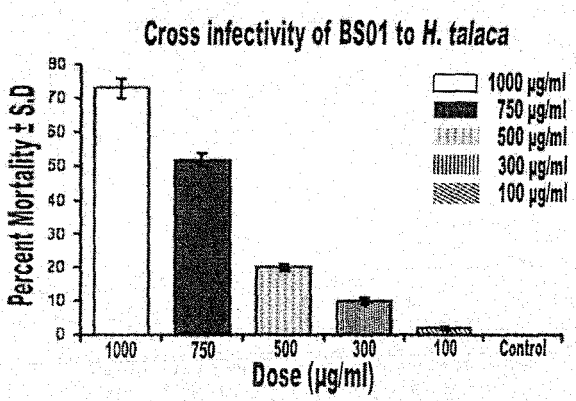
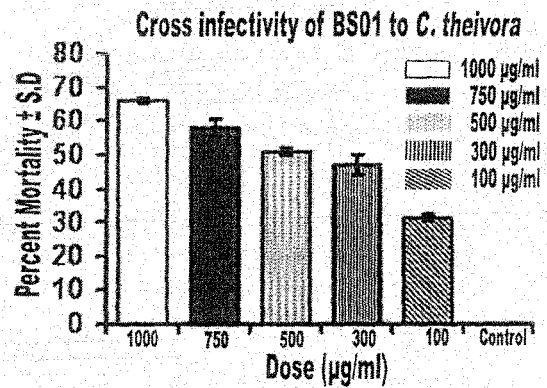


Graphical representation of table presented in page no. 56



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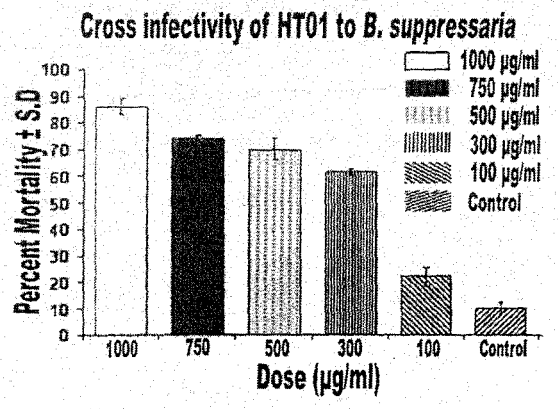

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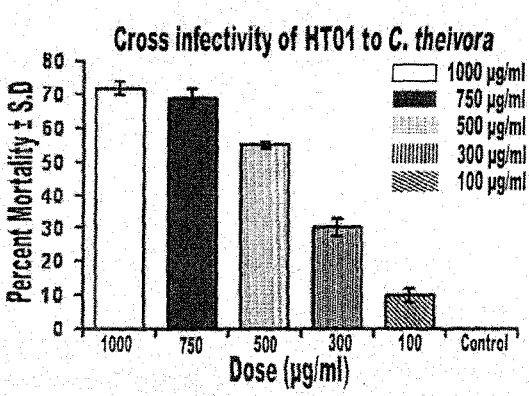

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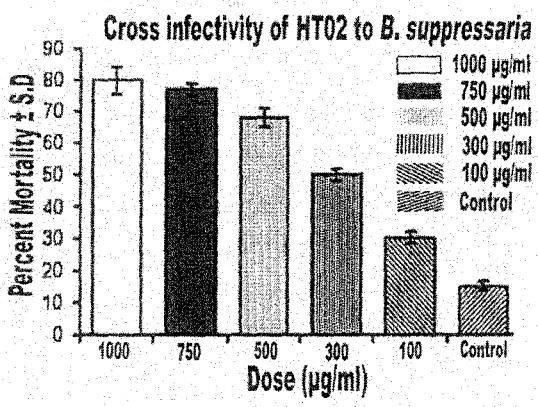

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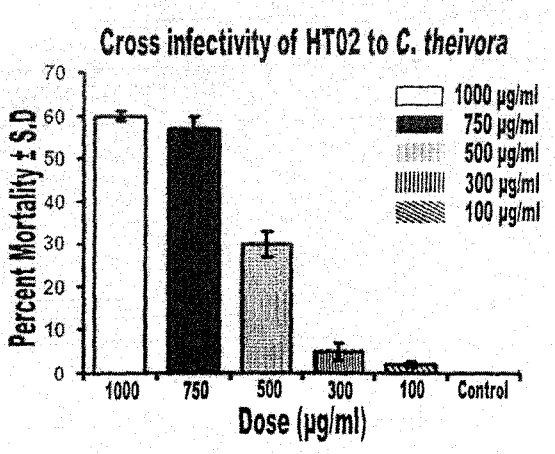

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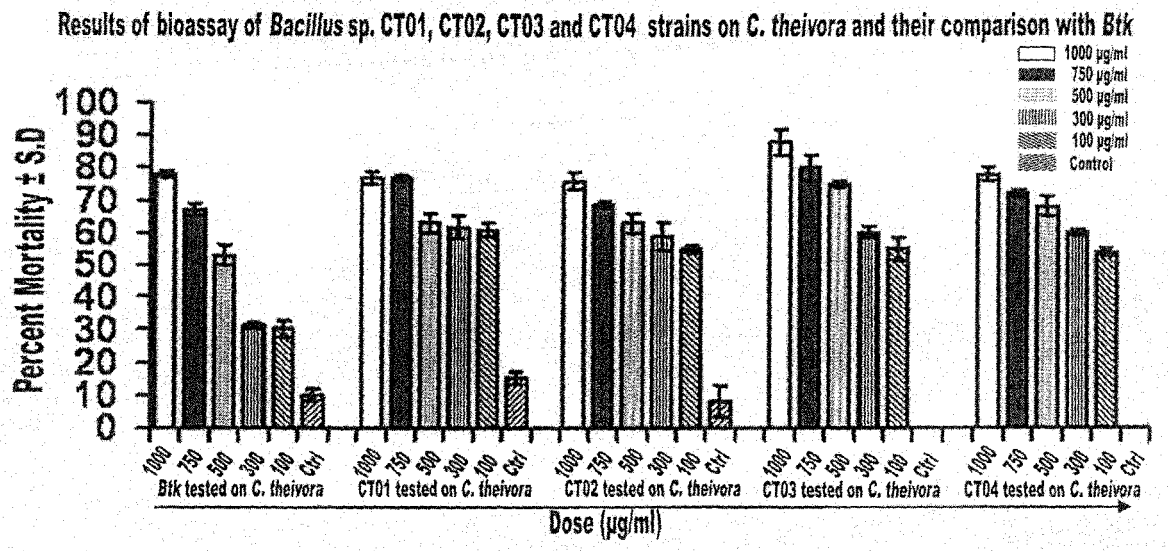

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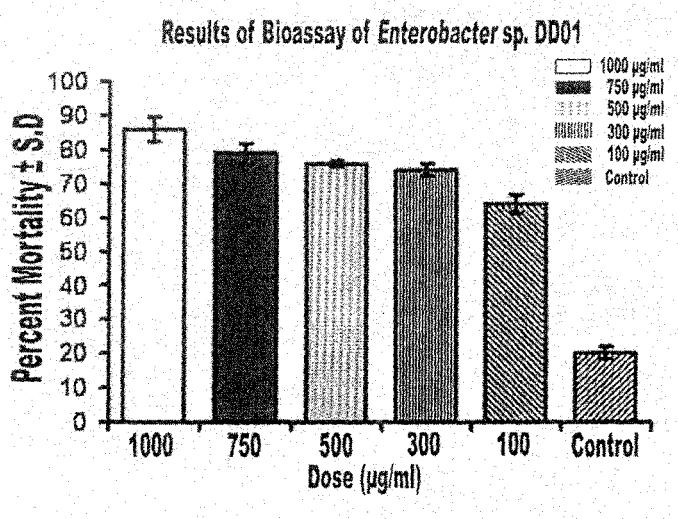

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Guerrero C, Pereira D and Net L (2007) Evaluation of the *Bacillus thuringiensis* based insecticide in the control of lepidopterous caterpillars on golf courses. *International Journal of Energy and Environment* 1(2): 106-108.

Ravi M, Santharam G and Sathiah N (2008) Ecofriendly management of tomato fruit borer, *Helicoverpa armigera* (Hubner). *Journal of Biopesticides* 1(2): 134-137.

Point no 3:

The observed selectivity of the bacteria, especially *Bacillus* to silkworm larvae needs clarification, as *Bacillus* is not species specific.

The bacterial strains selected to test cross infectivity to the lepidopteran tea pests of the same habitat and beneficial insect, such as silkworm is based on the most virulent strains (say of *Bacillus*) found to cause high mortality to the concerned tea pest from where it has been isolated. As the silk worm industry is running side by side with the tea industry, so before going into field study of the *Bacillus* strains it is necessary to know the cross-infectivity of the selected strains in silk worm population. So, the *Bacillus* strains were taken to cross-infect the silk worm population.

(Signature)

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