6. DISCUSSIONS

Isolation of bacteria from three sporadic tea pests

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

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

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

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

Physiological characteristics of the bacterial isolates

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

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

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

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

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

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

Biochemical characteristics of the *Bacillus* strains

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

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

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

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

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

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

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

Analysis of Crystal protein by SDS PAGE

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

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

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

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

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

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

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

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

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

Qualitative and Quantitative assay of whole cellular proteins of the isolates

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

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

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

Plasmid profiling of the bacterial strains

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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