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.