5. DISCUSSIONS

Population growth rates globally have so outstripped the linear rate of increases in food production that the Food and Agriculture Organization of the United Nations (FAO) estimated that 70% more food must be produced over the next four decades in order to nourish adequately a human population projected to exceed 9 billion by the year 2050 (FAO, 2009). The odds for attaining such an unprecedented increase, which would require the raising of the historically linear increases in annual food production by 37% (Tester et al., 2010), is substantially lessened by the consequences of climate change and variations on crop production systems (Beddington et al., 2011).

Providing adequate supply of food and improving the health of a rapidly increasing human population are two of the greatest challenges of today. The annual rate of food production increase in tropical developing nations is less than 1.0% while in most of these countries the population is growing at an annual rate of 2.0% (Youdeowei and Service, 1983). Thus there is a serious gap between food supply and demand. Crop losses to weeds, animal pests, pathogens and viruses continue to reduce available production of food and cash crops worldwide. Actual crop protection depends on the importance of pest groups or its perception by farmers and on the availability of crop protection methods. Actual loss rates show higher coefficients of variation than absolute losses in kg/ha (Oerke, 2000).
Without pesticides, an estimated two-thirds of all crops would be lost, depriving millions of peoples of food. Agricultural economists have estimated that in the absence of pesticides, production costs could increase by between 60 and 200% depending on the nature of the crop (Thygarajan, 1988). Of all the pesticides, insecticides are the most used. The proportion of pesticides used in 38 developing nations in 1973 was 60.2% insecticides, 30.4% fungicides and 3.4% herbicides (Youdeowei and Service, 1983).

Excessive use of insecticides for pest control has resulted in resistance development, pest resurgence, residual toxicity, environmental pollution, replacement of beneficial and non target species, outbreak of secondary pests and accumulation of harmful levels of pesticide residues in food and fodder (Devine and Furlong, 2007). To overcome these problems, eco-friendly approaches are being advocated. Synthetic chemical insecticides provide many benefits to food production and human health, but they also pose some hazards. Because of broad spectrum of toxicity many conventional insecticides raise concerns about human safety and the environment. Further, evolution of resistance to insecticides has occurred in more than 500 species of insects (Georghiou and Lagunes, 1991).

These problems with conventional insecticides are spurring the search for alternatives. One such alternative is the use of microbial insecticides- insecticides that contain microorganisms or their by-products. Microbial insecticides are especially valuable because their toxicity to non-target animals and humans is extremely low.
Compared to other commonly used insecticides, they are safe for both the pesticide user and consumers of treated crops. Microbial insecticides also are known as biological pathogens, and biological control agents. The most successful insect pathogen used for insect control is the bacterium *Bt*, which presently is 2% approximately of the total insecticidal market. *Bt* is almost exclusively active against larval stages of different insect orders and kills the insect by disruption of the midgut tissue followed by septicemia caused probably not only by *Bt*, but probably also by other bacterial species (Raymond et al., 2010).

*Bacillus thuringiensis* (*Bt*) is the oldest commercial biopesticide and has been in use under various formulations since 1938 (Weiser, 1961), the first commercial product being released in 1959. There is a long history of assessment of toxicology and environmental impact associated to the use of *Bt* insecticidal proteins, which led to the conclusion that these proteins are safe for non-target organisms and especially for mammals. The overall broad spectrum of *Bt* as a species associated with the narrow host range of each individual toxin also make this group of insecticidal proteins very attractive with respect to both efficiency and environmental safety.

*Bt* is the most promising and widely used microbial control agent because of its activity against important Lepidopteran and Dipteran pests and its relatively easy production in fermentors. *Bt* is an aerobic gram positive spore-forming bacteria characterized by the production of proteinaceous crystalline inclusions (crystals during sporulation.). A *Bt* strain can harbour more than one crystal and some crystals are
found to be comprised of several proteins (Endotoxins) are valued for their specific insecticidal activity and non-toxicity towards mammals. Variation of a single amino acid in the endotoxins can significantly influence the level of toxicity. Hence, it is essential to isolate and screen new *Bt* strains against pest insects. *Bt* is a major source for transfer of genes into plant genomes to develop insect resistant transgenic crop plants. Despite an earlier view that insects would not develop resistance to microbial insecticides, now it is realized that insect resistance to *Bt* can evolve rapidly under situations of selection pressure. However, the insect lines which developed resistance to a particular kind of *Bt* crystal proteins were found to be sensitive to a different kind of *Bt* crystal protein. Therefore, searches are continued throughout the world to isolate novel *Bt* crystal proteins, which might be effective killing the *Bt*-resistant insects (*Ferre et al.*, 1995).

India is considered as one of the centers of biodiversity (*Khoshoo*, 1994). This study is a part of the larger effort to sample biodiversity of *Bt* from Mizoram to contribute the diversity in India. Search for novel *Bt* strains may lead to the discovery of additional insecticidal proteins with higher toxicity and/or wider spectrum (*Ramalakshmi and Udayasuriyan*, 2010; *Martin and Travers*, 1989). New variants of the already known *cry* gene subgroups could encode crystal proteins with significant difference in the level and spectrum of toxicity due to variation in their sequences (*Xue et al.*, 2008). The soil samples of Mizoram (one of the biodiversity hotspots) may yield new isolates of *Bt* with novel *Cry* proteins which could be used for control of insect pests. In the present study, 55 soil samples from five different habitats of the Mizoram were used as a source material for isolation of indigenous *Bt* strains.
The taxonomy of *B. thuringiensis* has been the subject of study since the late 1950s. Methods of identifying *Bt* varieties were proposed, based on morphological and biochemical characterization, using conventional microbiological techniques (Heimpel and Angus, 1958). H-serotyping, the immunological reaction test to the flagellum antigen (de Barjac and Bonnefoi, 1962; 1973) was also developed early and is still widely accepted for classification and identification of *Bt* (de Barjac and Frachon, 1990; Lecadet *et al.*, 1999). Morphological and biochemical characterization of *Bt* isolates from Mizoram resulted in proper identification and classification, in accordance with the standard strains used, and they produced oval, bipyramidal and spherical crystals. The present study is the first scientific documentation of *Bt* from the soils of Mizoram.

Results of the present study showed that about 52.72% of the 29 samples were positive for *Bt* and yielded 107 isolates. Earlier studies reported varied frequency for isolation of *Bt* from soil samples ranging from 3 to 85% (Ramalakshmi and Udayasuriyan 2010; Martin and Travers 1989; Wang *et al.*, 2003). Moderate frequency (52.72%) for isolation of *Bt* from the five different habitats, in the present study, may be due to large amount of nutrients in the soil itself, allowing optimum survival and enrichment in the soils. Soil is very important source of *Bt* strain providing a large genetic resource for its use in the development of bioinsecticide to control insect pests (Quesada-Moraga *et al.*, 2004). The *Bt* index from Mizoram ranged from 0.010 to 0.015 in the soil samples studied. Extreme values were frequently reported in several studies to range from 0 to 0.2 in United States (DeLucca *et al.*, 1981), 0.2 to 0.5 in New Zealand...
(Chilcott and Wigley, 1993), 0.75 in Bangladesh (Hossain et al., 1997), 0.009 to 0.380 in Thailand (Martin and Travers, 1989) and 0.15 to 0.18 in Western Ghats, India (Ramalakshmi and Udayasuriyan, 2010). Vilas-Bôas and Manoel (2004) suggested the Bt index may be a consequence of biotic environmental factor, e.g., microorganism in the soil, the type of insect commonly found in the area, or the vegetation besides, abiotic factors such as the pH, texture, oxygen and nutrient availability, temperature, and humidity.

In the present study, 107 of the 8,676-stained bacterial colonies observed through phase contrast microscopy showed the presence of crystalline inclusions, and were characterized into three major groups viz., spherical, oval and bipyramidal. These findings differed from the earlier reports (Bernhard et al., 1997; Ramalakshmi and Udayasuriyan, 2010; Martin and Travers, 1989), wherein strains with bipyramidal (46%) and cuboidal (26.9%) crystals were predominant. Differences observed in the morphology of crystalline inclusions of Bt suggested presence of diversity in the Bt isolates of Mizoram.

Grouping of Bt isolates according to crystal protein(s) profile studied by SDS-PAGE will give a prelude for the presence of diversity in cry genes. The lepidopteran-active cry1 (130–140 kDa), lepidopteran and dipteran-active cry2 (65-75 and 135 kDa), coleopteran active - cry3 (66-73 and 70-75 kDa), dipteran-active cry4 (125-145, 68, 72, 78, 128, 130-140 and 135 kDa), cry10 (80 kDa) and cry11 (67-94 and 80 kDa), cry 5-9 gene (129, 73, 75, 35 and 38 kDa) show toxicity to different insect orders were
described by Crickmore et al.,(2010). Therefore, analysis of crystal proteins(s) profile could be useful to predict the presence of cry genes. In the present study, 60 of the 107 isolates are having 135 and/or 65 kDa proteins suggesting the presence of genes related to cry4 and cry9 families. Other isolates showed that the presence of 43 (group I) or 30 (group II) or 43 and 30 (group III) or 17 and 60 (group VII) or 19 (group VIII) or 27 and 95 (group IX) or 14 and 25 (group X) or 22 and 106 (group XI) kDa proteins indicating the presence of other novel cry genes also. Among the eleven groups identified, groups IV-VI are more predominant (56.07%) and group VII-XI are novel Bt isolates (22.42%). These results led us to suggest the presence of diversity in Bt isolates of Mizoram.

Cry proteins that could be of particular interest for this study are: Cry4 and Cry9 since they have been reported as effective against dipterans especially mosquitoes (Schnepf et al., 1998) and most abundant in Mizoram soils. The characterizations for most of the Bt collections were based on bioassays against different insect larvae without identification of the Cry genes present in the Bt strains. In this study, 25 isolates were selected for toxicity, Cry gene and protein profiling. All the Bt isolates showed 60 - 100% mortality, low LC50 values and possess Cry 4 and/or 9 genes.

Optical Density is an indication of cell numbers in liquid media, enables the time necessary to reach stationary phase and crystal formation to be determined. The growth curve analysis was performed to determine the time required by the bacterium to reach its vegetative or exponential phase in a batch culture with shaking. In growth curve
studies, culturing conditions such as incubation time (24-48 h) and growth medium (LB agar) significantly influenced final yield. Further, log phase was varied between Bt cultures which is the major variable that determines the population size, production of cry toxin and their toxicity (Crickmore et al., 2010). From the growth curve it is evident that the bacterium remains in its vegetative or exponential phase from 21-42 h. The result of this experiment was very much in accordance to the growth pattern demonstrated by Sattar et al., (2008) for a different Bt strain. Thus the protein was harvested from the culture supernatant after 24 h of culture, expecting maximum yield of vegetative proteins secreted by the Bt strain. The time taken to harvest the fermentation varied among the Bt cultures used.

The cry gene and protein profiling and toxicity (mortality, LC$_{50}$ and LT$_{50}$) of Bt isolates significantly varied within habitats. The differences in toxicity of Bt isolates to C. tritaeniorhynchus may be related to the composition of cry gene and protein, crystal production and their toxic potential. Bt isolates SC1 and HP7 have Cry4 and Cry9 genes related to high toxicity (low LC$_{50}$, lesser LT$_{50}$, lesser time for crystal production and harvesting and 100% mortality), while CHTP1, LL1, CH1, RD2, LP4, LT1, SK1, SL2, SR1, WP1, LP2, LP3 and SH1 isolates have one to three cry gene combinations (Cry4/Cry9/Cry3,9/Cry4,9/ Cry1,2,4/ Cry1,4,9 and Cry2,3,9) and showing high LC$_{50}$, longer LT$_{50}$, longer harvesting time and 60 - 75% mortality. Distribution of cry gene and proteins and their toxicity to C. tritaeniorhynchus varied between Bt isolates which may be influenced by habitat, interaction between cry genes, expression of cry gene, crystal morphology and production and bacterial growth conditions. The presence of different
cry genes in the same *Bt* strain has been reported, for example Aronson (1994) and Ben-Dov *et al.* (1997) reported the presence of *cry*1 and *cry*3, *cry*8, or *cry*7 genes in the same *Bt* strain. Chowanadisai *et al.* (1995) found highly effective isolates for controlling *Aedes* mosquitoes larvae, namely S-KB1802, S-KB1001 and S-KB2701, which showed LC50 at 1.28x102, 3.59x102 and 9.80x102 spores/ml, respectively. The importance of the isolation of native strains of *Bt* in the mosquito management program in Mizoram is important based on the findings.

Jansen *et al.* (1997) reported Cry9 was toxic to *S. litura, S. exigua, H. armigera* especially *P. xylostella* that is resistant to *cry*1 gene groups. The toxicity of *Bt* did not depend on *cry* gene content only because factors other than Cry proteins may contribute to toxicity as well as spore interaction with crystal protein and the other soluble toxins such as β-exotoxin (Porcar *et al.*, 2000). The identification of known *cry* genes in the *Bt* strains is important, since the specificity of action is known for many of the Cry toxins. This fact allows the possibility of selecting native strains that could be used in the control of some targets and of selecting strains with the highest activity. The PCR screening is a rapid method for detecting and differentiating *Bt* field strains by their PCR product profiles and for predicting their insecticidal activities in order to direct them for subsequent toxicity assays against lepidoptera, coleoptera and diptera.

RAPD analysis of *Bt* isolates in Jordan revealed high polymorphism between isolates which is in accordance with the present study (Sadder *et al.*, 2006). RAPD-PCR assay has been optimized that discriminate *Bt* isolates from nine different habitats soil
covering five districts of Mizoram. All the studied isolates showed a diverse RAPD patterns and were different from each other in relation to habitats, toxicity and type of cry gene present. High polymorphism was observed between Bt isolates which was authenticated through high PIC, RP, EMR and MI values. Further, three major clusters were identified through dissimilarity analysis. No relationship found between the type of cry gene in Bt and their toxicity against mosquitoes. Markers generated by RAPD were used to fingerprint and elucidate phylogenetic relationships of many microorganisms such as Aphelenchus avenae (Ali et al., 1999); Renibacterium salmoninarum (Grayson et al., 2000); Eschericia coli (Aslam et al., 2003); Loctobacillus plantarum (Elegado et al., 2004); Serratia marcescens (Enciso-Moreno et al., 2004); Xanthomonas axonopodis (Khoodoo and Jaufeerally-Fakim, 2004) and Staphylococcus aureus (Casey et al., 2007).

The advantage of RAPD analysis in this study is that it covers the entire genome; therefore it provides sufficient information about differences that might be present inside the genome. Williams et al., (1990) showed that RAPD markers cover the entire genome, revealing coding or non-coding regions, repeated or single-copy sequences. Also, Schnell et al., (1995) reported that the arbitrary nature of the primer resulted in amplified DNA products representing random samples of the entire genome. This interpretation was reinforced by Michelmore et al., (1991) who reported that polymorphism in RAPD profile might be resulted from base changes that alter primer-binding sites. Similarly, Lu et al., (1996) and Martin et al., (2000) revealed that polymorphism might be due to structural changes in the genomic DNA that alter the
distance between two annealing sites, delete an existing site or insert a new one, insertion of a DNA segment that render priming sites to distant to support amplification or insertions that increase the distance between two priming sites without preventing its amplification. Yang and Quiros (1993) reported that the intensity of DNA bands depends on the starting copy number of a particular DNA sequence within the genome. Therefore, the differences in band’s intensity could be interpreted on the basis of alterations of some DNA sequences.

Konecka et al. (2007) used RAPD analysis to estimate phylogenetic relationships among twelve Bacillus thuringiensis strains isolated from intestinal tracts of Cydia pomonella larvae. The result indicated a tendency of bacterial strains to cluster according to their source which in similar to the results of the findings in the present study. The Bt isolates from agricultural soil, aquatic and barren land formed three separate clusters and branched out according to their source or habitat. Molecular typing methods, such as Arbitrary Primer- PCR technology (Brousseau et al., 1993), DNA re-association measurements (Nakamura, 1994), ribosomal RNA gene b restriction fragment length polymorphism (Priest et al.,1994; Akhurst et al., 1997), ribosomal RNA gene intergenic spacer sequences comparison (Bourque et al., 1995), and DNA-colony hybridization and random amplified polymorphic DNA (RAPD) analysis (Hansen et al., 1998), have also been applied to limited numbers of B. thuringiensis strains.
Genotype-based molecular typing methods, 16s rRNA gene polymorphism studies (ribotyping), has proved to be very effective as a molecular taxonomic tool for estimating chromosomal genetic diversity and relationships among various bacterial species and subspecies (Saunders et al., 1988; Grimont and Grimont, 1991; Hernandez et al., 1991; Williams and Collins, 1991; Jacquet et al., 1992; Mugnai et al., 1994; Okwumabua et al., 1995). In the present study, minor sequence differences among the 16s rRNA gene sequences of B. thuringiensis strains was observed. Phylogenetic analysis using 16s rRNA sequence showed that it is highly conserved; and is a reliable method for species identification as well as authenticity of its use in molecular biology.

16S rRNA gene restriction band pattern analysis is not subjected to the constraints associated with the B. thuringiensis serotyping system. Using the B. subtilis 16S rRNA gene as a probe, Priest et al. (1994) examined 43 B. thuringiensis strains from 10 serovars by ribotyping with Hind III. The diversity of the 16S rRNA gene restriction patterns was higher for B. thuringiensis than for any other species examined by same technique (Verger et al., 1987; Saunders et al., 1988; De Buyser et al., 1989; Aquino de Muro et al., 1992; Jacquet et al., 1992). Bourque et al., (1995) examined 24 strains of B. thuringiensis belonging to seven serovars and two closely-related species, B. anthracis and B. cereus, by comparative analysis of the 16S to 23S ribosomal intergenic spacer sequences.

In conclusion, high Bt index was recorded in the shifting cultivation while lowest frequency was observed in the forest habitats of Mizoram. The Bt isolates of Mizoram
exhibited eleven different types of crystal protein profile revealing the molecular diversity of this bacterium in different habitats of Mizoram. *cry*4 and *cry*9 genes were found to be the most abundant in *Bt* isolates of Mizoram soils, which have strong activity against dipteran insects (*C. tritaeniorhynchus*). Genetic characterization of *Bt* isolates revealed polymorphism and exhibited diversity according to their habitat. Due to their distinctive characteristics, CHP5, RRK, HP7, RP, LP1, LP4, SL1, SC1, CHTP isolates are impressive candidates for the development of new larvicidal formulations. The isolation of native strains with activity against dipteran and lepidopteran pests, gives us new tools to be introduced into the pest management program.