2 REVIEW OF LITERATURE

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

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

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

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

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

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

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

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

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

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

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

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

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

2.3 Entomopathogenic bacteria: categories and strains

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

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

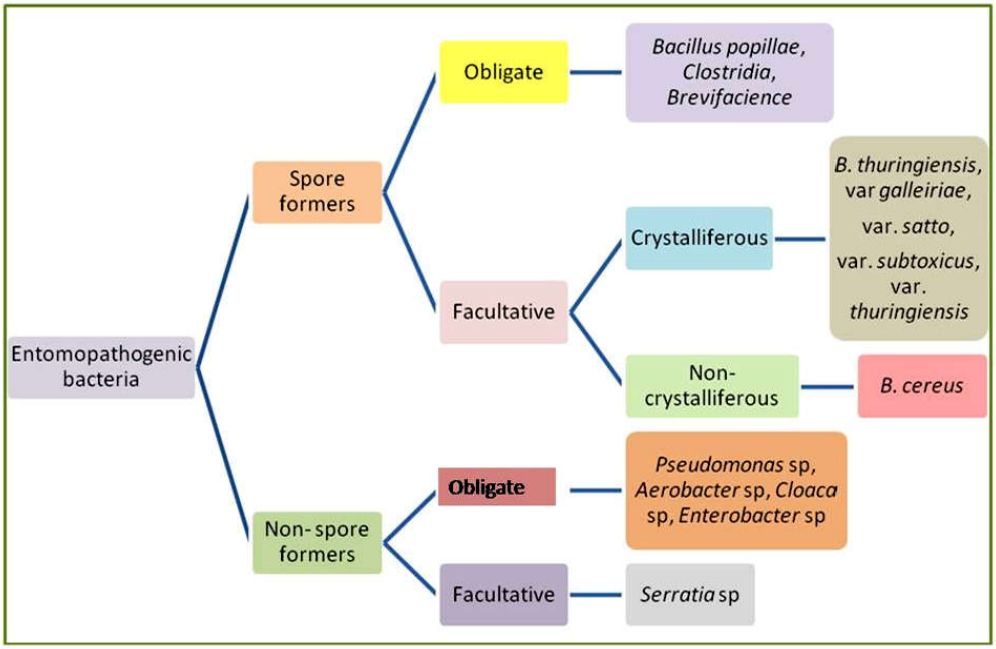

Fig. 2.1: The classification of entomopathogenic bacteria

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

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

2.4.1 *Bacillus thuringiensis*: a historical perspective

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

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

2.4.2 Natural occurrence of *B. thuringiensis*

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

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

2.4.3 Mode of action on target insects

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

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

2.5 *Bacillus thuringiensis* as biocontrol in tea pests

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

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

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

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

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

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

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

2.6 Resistance to *Bacillus thuringiensis*

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

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

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

2.7 Need for searching novel and improved entomopathogenic bacterial strains

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

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

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

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

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

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

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

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

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

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

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

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

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

2.10 Screening of *Bt* toxic gene by PCR

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

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

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