1. Introduction:

World population is expected to double by the year 2050. Ninety seven percent of this population increase will occur in the developing countries, including Asia (Swaminathan 1995). The 1995 Asian Development Bank's policy paper on Agriculture (ADB 1995) reports that currently 700 to 800 million of the World's one billion poor people live in Asia and Pacific region and that about 500 million live in absolute poverty. A very large population of the poor people is totally dependent on agriculture. Thus, for the future, one of the forbidding challenges is how to increase economic growth. With increasing population, food production will have to be doubled or tripled by middle of the 21st century on the same existing area of cultivated land, practicing sustainable agriculture that coincidentally conserves natural resources. Agricultural research, new technologies and improved seeds will continue to be prerequisites for increasing agricultural productivity and for enhancing income of the farmers who can produce more food for the billions of people. Consequently, increasing productivity per unit of cultivable land represents the only major opportunity for increasing global production of food, feed and fiber. Quantum leaps in agricultural production by more sustainability using natural resources are required to feed the burgeoning population. The productivity of wheat and rice increased through "Green Revolution" following the traditional technology, but it will have to be supplemented by modern biotechnology applications to meet the forbidding food challenge of the 21st century.

There are possibly numerous sustainable ways that agricultural productivity can be raised. Many technologies need to be deployed concurrently to counteract the negative impacts of the current degradation of the agro-ecosystem. In sum, elements that can play

major roles are biological fertilizers, soil and water conservation, biodiversity conservation, improved pest control, and last but not the least, changes in land ownership and distribution. Application of biotechnological measures integrated into traditional agricultural systems hold distinct promises to augment conventional agricultural production capacity in order to bring in sustainability.

Biotic stresses in crop production due to insects, are estimated to reduce global crop production by 14% (James 1981, 1996; James et al 1991). A heavy infestation by certain insects in a crop sometimes can even result in loss of crop yield to 40% or more. The insecticides used in the form of chemical pesticides in 1994 on four major groups of crops namely fruit and vegetables, cotton, rice and maize have incurred expenditure of about US$ 8.1 billion to combat yield loss. However, diversity of the use of chemical pesticides is often not cost effective. It also shows adverse effects on non-target organism including animals, birds and human beings. Moreover, resistance to chemical insecticides has been seen to occur in several insect species. Management of such resistance among pests remains a challenge, and new methods of controlling such species are needed implement. This prompted us to search for alternative ecofriendly, cost effective and sustainable insect control agents. The insecticidal toxin proteins obtained from the *Bacillus thuringiensis* (*Bt*), a gram-positive soil bacterium, turned out to be a key player.

The name *Bacillus thuringiensis* dates from 1911, when Berliner isolated a crystal-containing organism from diseased flour moth larvae. The use of *Bt* as a biopesticide was discovered in the first decade of the 20th century (Berliner et al 1911). However, it took 50 years before *Bt* became a widely used biopesticide with its registration in the USA in 1961. But even today, less than one percent of all pesticides used in the USA each year are *Bt* based

products. Over half of the *Bt* biopesticides are used in, the USA out of a total worldwide market of biopesticide of US$24 million in 1980. This market grew to US$107 million in 1989; at annual growth rates of 11% (Feitelson et al 1992). By the beginning of 1990s, introduction of new *Bt* strains and formulations and development of recombinant *Bt* products have furthered the growth of the *Bt* market, mainly by extending the insect host range of the microbial products and enhancing the insecticidal potencies of the toxins. In forestry, *Bt* has effectively replaced chemical insecticides and has become established in control programs against defoliator larval moths in Europe and North America (van Frankenhuyzen 2000). In Israel, since 1987 *Bt* has effectively replaced chemical insecticides against the insects in pine forests (Navon 2000). Over 90% of the biopesticide sales are one single product type, the *Bt*-based products. Two companies, Abbot Laboratories and Sandoz Corporation, dominated the market with approximately 70% of the total production worldwide. The difference is produced by about 30 companies (Lisanski 1992), leading to a range of over 100 *Bt* product formulations. Commercial formulations of *Bt* spore crystal toxin mixture (such as Thuricide™ and Dipel™ as biopesticide) have been in use for more than 4 decades for crop protection. Most of them are based on one protein, although some contain as many as 5 different *Bt* toxins.

The advantage of *Bt*-based biopesticide lies in:

i) Production of stable spores containing the toxin protein for use in conventional pest control application and is technically simple.

ii) *Bt* toxin is totally biodegradable and does not show toxicity towards plants and animals including human beings and also to other friendly insects (Bruges 1982; Orr et al 1997; Pilcher et al 1997; Fitt et al 1994).

Bt produces insecticidal crystal proteins (ICPs) during sporulation (Höfte and Whitely 1989). Upon sporulation, *Bt* produces cytoplasmic inclusions containing one or more ICPs (Knowls 1994). Most ICPs are synthesized intra cellularly as inactive protoxins and they spontaneously form small crystals, approximately 1 μM in size. Many *Bt* isolates, produce proteins that crystallize in different shapes, such as cuboidal and flat square crystals. *Bt* isolates also produce bipyramidal crystals. These are typically 130-140 kD in size. Some reports also indicate presence of DNA fragments in the crystals, but their role in toxicity if any, is unknown (Bietlot et al 1993). Upon ingestion by the susceptible insects, the crystals are solubilized in the insect gut juice. The solubilized protoxins are activated by gut proteases producing a protease resistant core, representing the active toxin. During activation by proteases, peptides are removed from both N and C termini of the protoxins. Among the high molecular mass Cry protoxins, proteolytic activation removes nearly half of the molecule from the C terminus. The toxin binds to high affinity sites on the surface of the midgut epithelial cells (Hoffmann et al 1988; van Rie et al 1989,1990; Masson et al 1995a, 1995b). These cells eventually swell and cause loss of gut integrity and result in larval death within 1-2 days (Knowles and Ellar 1987).

1.1. Insect specificity and variability of *Bt* toxin:

Höfte and Whitely in 1989 proposed a classification scheme for *cry* genes. At that time, there were about 40 *cry* genes that were cloned and characterized. The genes were classified into four groups based on insect specificity and nucleotide sequence similarities. The *cry*I genes encode 130-140 kD proteins that are exclusively active against Lepidopteran insects; *cry*II genes encode 70 kD proteins that are sensitive to both Lepidopteran and Dipteran insects; *cry*III gene products are active on Coleopteran species and their proteins are about 70 kD; *cry* IV gene products of both 70 kD and 130 kD crystal proteins are highly

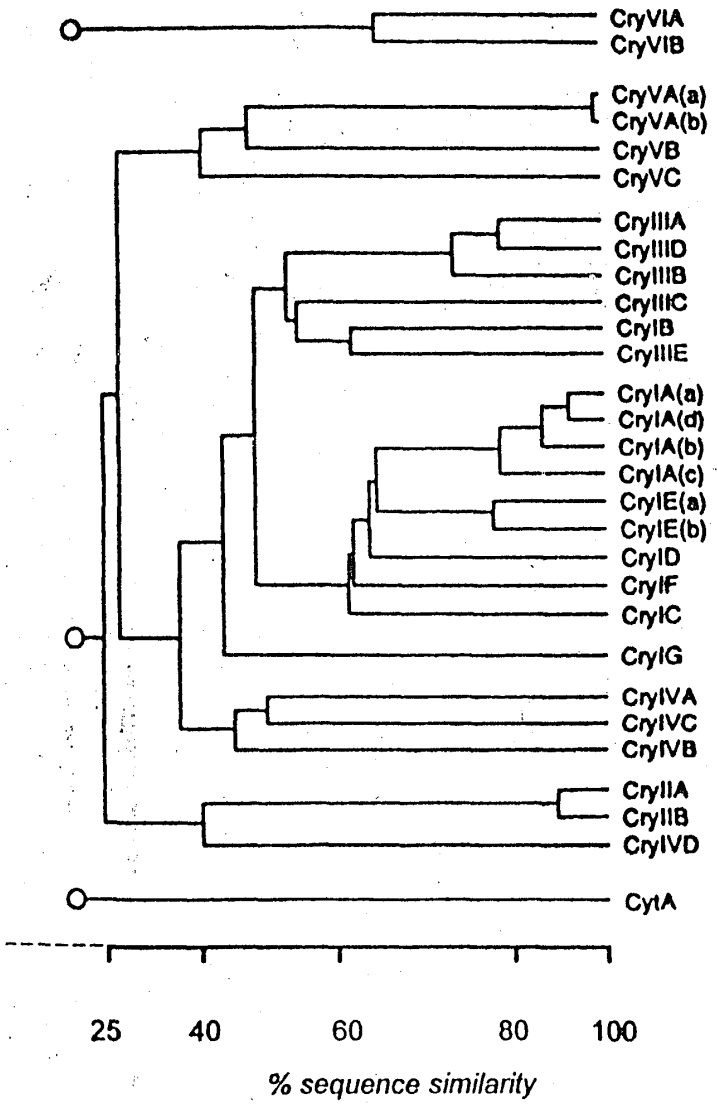
active against Dipteran insects. In addition to that several *Bt* subspecies are known to produce cytolytic crystal protein that has a molecular mass of approximately 27 kD. Recently, some *Bt* proteins that are active against Protozoa and Nematodes have also been discovered.

The classification scheme as described by Höfte and Whitely (1989) has faced with problems in handling an ever-growing list of *cry* genes. Alignment of *cry* gene sequences provide insight into the evolutionary relationships between the different *cry* gene types (Fietelson et al 1992; Crickmore et al 1996, 1998). A dendogram (Fietelson et al 1992) reveals clusters of crystal proteins that are closely related in the evolutionary process (Fig-1a). The degree of heterogeneity between CryIA and CryIG, in fact, is almost identical to the difference between CryIA and CryIV. Some Cry proteins eg; CryIB and CryIG have a higher degree of heterogeneity than others. Till now there are at least more than 130 kinds of *Bt* toxins known to be toxic to different insects, nematodes and protozoa (http// www.epunix.biols.susx.ac.uk/Bt index). Different *Bt* toxins show diversity (Fig-1b) in their insect specificity i.e., one type of toxin protein is active in one or a few insect species. Based on their range of insecticidal activity and amino acid homology (Höfte and Whitely 1989), they have been further classified. Insect specificity of different *Bt* ICP is determined by the toxin-receptor interaction in the insect midgut (van Rie et al 1990; Gill et al 1992).

1.2. Structure of *Bt* toxin molecule:

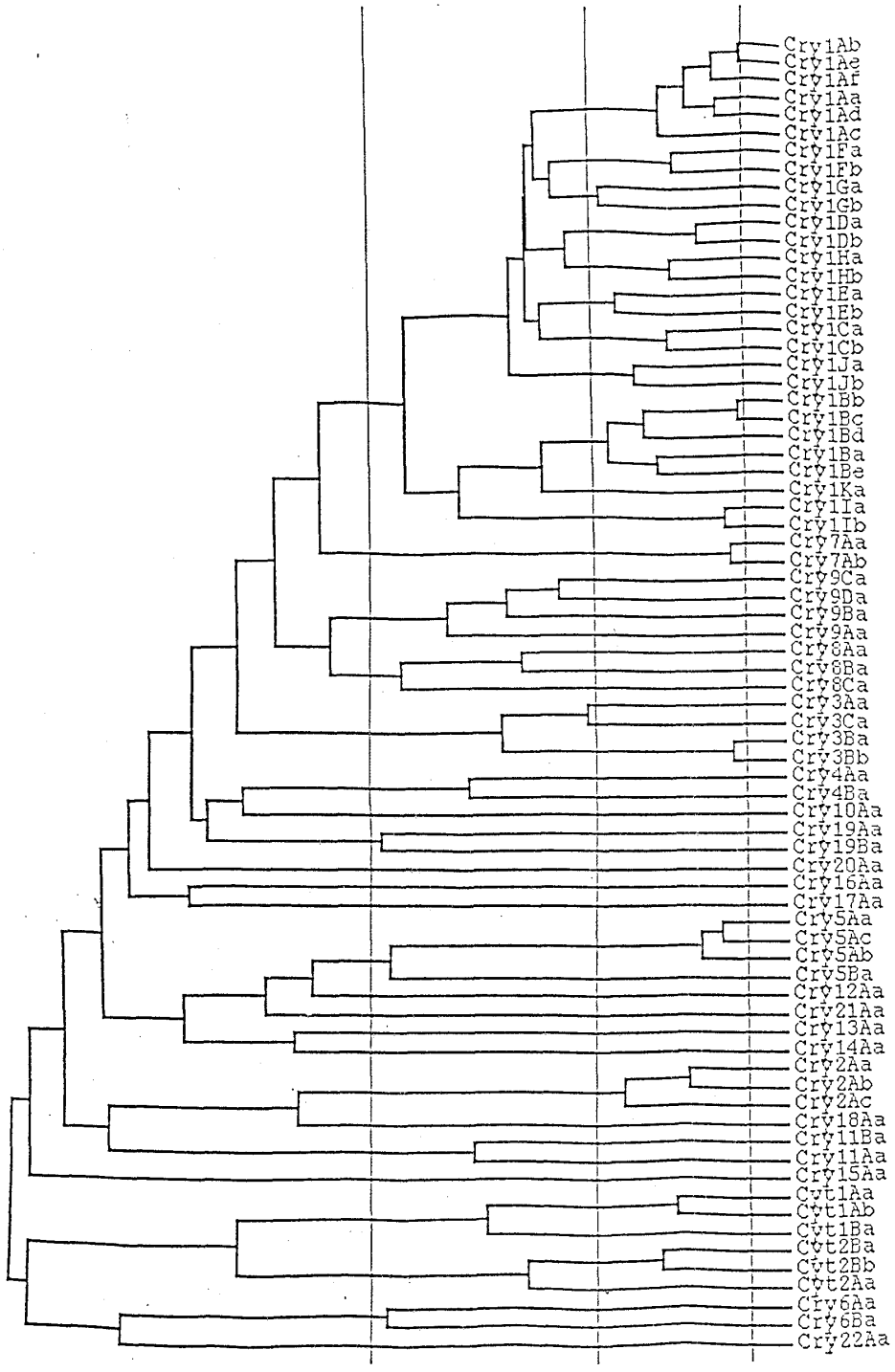
Information on toxin structure has come mainly from structural and mutational studies. Three-dimensional structures of CryIAa (Grochuliski et al 1995) and CryIIIA (Li et al 1991) toxin molecules have been developed from X-ray crystallographic studies. These two toxins are structurally similar, although they differ in their amino acid sequences and their host specificity. CryIAa toxin is lepidopteran-specific, whereas

Fig-1a. Amino Acid Sequence Similarity of the *Bt* Proteins



Source: Feitelson *et al.* (1992).

Fig-1b.Dendogram showing sequence similarity of different *Bt* proteins



http://epunix.biols.sus..._Crickmore/Bt/tree.html

http://epunix.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/tree.html

CryIIIA toxin is coleopteran-specific. Both proteins consist of three functionally distinct domains designated as domain I, domain II and domain III.

Domain I: In CryIAa toxin domain I extends from residue 33 to 253 from the N terminal end. It forms a bundle of eight antiparallel α-helices. This domain is rich in hydrophobic and amphipathic amino acids (Grochulski et al 1995). It has been shown that the helices of Domain I are responsible for the formation of voltage dependent ion channels in the lipid bilayer of epithelial membrane of the insect midgut (Walters et al 1993; von Tersch et al 1994; Cummings et al 1994).

Domain II: The domain II in CryIAa toxin is made up of residues from 265 to 461. It consists of three antiparallel β-sheets and two short α-helices. Mutational studies implicate that this domain is responsible for binding on specific receptors present on the midgut epithelium (Gill et al 1992; Knowles 1994; Schnepf et al 1990; Lu et al 1994).

Domain III: Domain III is a β–sandwich of two antiparallels, highly twisted β-sheets and comprises residues from 463 to 609. The role of domain III is unclear although recent studies indicate that this domain may also participate in receptor recognition and can influence insect specificity (Bosh et al 1994; Masson et al 1994).

Toxin domains are closely packed together in CryIAa. Domain I and II are packed by van der Waals forces, hydrogen bond and electrostatic attraction. Four salt bridges are formed at the interface. Such salt bridges keep the protoxin crystals insoluble over a wide range of pH. It was also found that at these pH ranges most of the hydrogen bonds and salt bridges remain unchanged. As a consequence, there are no or little conformational changes in their structure in the insect midgut (Grochulski et al 1995). Association of domain I and domain III involves a smaller buried surface. The contacts are primarily with these two hydrophobic domains. In addition to that, one bridging bond is also

formed. No salt bridges are found between these two domains (Grochulski et al 1995). A very short linker connects domains II and III. The interactions are mostly hydrophobic but there are also hydrogen bonds between the two domains and one bridging water molecule buried in the interface. There are no salt bridges between these domains (Grochulski et al 1995).

Sequence homology level between CryIAa and CryIIIA (Li et al 1991) shows structural similarity between them. Both are built from 3 domains and corresponding domain shows the same topological folds. Domain III shows highest similarity followed by domain I, while the largest divergence is observed in domain II. Amino acid identity between domain I of CryIAa and CryIIIA is 35%. Domain II is where the CryIAa and CryIIIA molecules are the most divergent. The amino acid identity of 23% is much lower than for the α-helical domain. The most structurally similar domains of CryIAa and CryIIIA are domain III. They show highest (41%) amino acid similarity (Grochulski et al 1995). The domains associated in a similar manner in CryIAa and CryIIIA (Goruchuliski et al 1995). This structure is more or less considered to be common for all the Cry toxins as revealed from mutational studies.

1.3. Mode of action of *Bt* toxin:

Mode of action of *Bt* δ-endotoxin involves a cascade of events. These are solubilization of the crystal, activation of the toxins by gut proteases in high alkaline pH and recognition of a binding site on the midgut brush border membrane epithelial cells, followed by the pore formation, membrane transport disruption, and cell lysis, leading ultimately to insect death (English et al 1992; Gill et al 1992; Knowles et al 1993; Schnepf et al 1998).

1.3a. Receptor binding:

Reciprocal hybrid genes between closely related toxins of differing specificity's for several Cry toxins have indicated that the receptor specificity-determining region lies primarily in domain II (Ge et al 1989, 1991; Widner et al 1989; Schnepf et al 1990; Masson et al 1994). Further studies have indicated that the receptors for crystal protein represents aminopeptidase N that is linked to the luminal side of the midgut epithelial cells (Denolf et al 1987; Gill et al 1995; Knight et al 1994; 1995). Site directed mutational studies found that 365 to 371 of CryIAa toxin are essential for binding to the membrane of midgut epithelial cells of *Bombyx mori* (Lu et al 1994). In the three-dimensional structure, this segment forms a loop and is highly mobile in the crystal. This loop is not involved in crystal contacts and has few interactions with the rest of the domain II. The intrinsic flexibility of this loop does play an important role in receptor recognition (Grochuliski et al 1995). The loop structure of CryIAa is structurally dissimilar to the loop structure of CryIIIA (Grochuliski et al 1995; Smith et al 1994) and this could be one of the reasons for their differences in host specificity. CryIAa is lepidoptera specific and CryIIIA being coleoptera specific. The CryIIA toxins are sensitive to insects belonging to lepidoptera and diptera. The dual specificity of CryIIA, as distinct from the lepidoptera specificity in the closely related CryIIB is determined by residues 307-382 of their sequences (Widner et al 1990). On the other hand the specificity-determining region of CryIAc toxin exists in three different regions of domain II (Lee et al 1997).

Experiments in which different fragments from closely related toxins were exchanged, found that specificity determining and receptor binding domains were present on the domain II of a number of toxins (Ge et al 1989,1991; Lee et al 1992; Liang et al 1994; Masson et al 1994; Tabashnik et al 1996; Widner et al 1990). Site directed

mutagenesis experiments have further stressed the importance of domain II surface exposed loops situated at the apex of the molecule in receptor recognition (Lee et al 1996; Lu et al 1994; Rajamohan et al 1995, 1996, 1996; Smith et al 1994; Wu et al 1996). Domain III is also involved in specificity since mutations in this domain which affect toxicity and binding have been described (Aronson et al 1995). These observations sufficiently indicate that the interactions and involvement of both domain II and domain III play functional roles in receptor recognition.

1.3b. Pore formation:

The common mechanism of epithelial cell disruption by δ–endotoxin of widely different specificities is believed to be the formation of lytic pores of 10-20 A^0 diameters in the insect membrane (Knowles et al 1987). Domain I is thought to be responsible for pore formation in the epithelial cell membrane. A number of mutations in domain I abolish or reduce the toxicity of the protein without affecting its binding properties significantly (Ahmed et al 1990; Chen et al 1995; Hussein et al 1996; Wu et al 1992). N-terminal fragments of CryIAc (Walters et al 1993) and CryIIIB (von Tersch et al 1994) have been shown to form channels in lipid bilayer membranes. The active toxin binds to the specific receptor proteins on the epithelial plasma membrane of the insect midgut. This triggers a conformational change in the toxin, helping the insertion of domain I into the epithelial cell membrane (Ahmed et al 1990). Toxin binding on BBMV has been shown to become irreversible within a short incubation time, implying a membrane insertion step following the initial binding. The domain I of the bound peptide inserts itself to the plasma bilayer forming channels that cause rapid ion movement from the epithelial cells leading to osmolysis causing larval death (van Rie et al 1989).