1. Introduction

1.1 History of Agriculture

Agriculture began to developed over the 10,000 years ago in during evolutionary world. Peoples everywhere have discovered the food value of wild plants and animals. They domesticated and bred them (Harris, 1996). Wheat, rice, barley, corn, rye sugarcane and sugar beet had the most important cultivated plants (Gupta, 2004; Fuller,2006). In Neolithic culture era, agricultural sites occupied by such people were located in southwestern, southeastern Asia, Africa, Europe (Gangal et al., 2014). Early places of agriculture have also been identified in the Yellow River area of China (Singh and Yadava, 2003); the Indus River valley of India and Pakistan (Krebs and Krebs,2003). As per scientific technique - carbon dating, barley, and wheat were domesticated in the Middle East in the 8000 BC; rice and millet in China and southeastern Asia by 5500 BC, Legumes found in Macedonia and Thessaly were reported as early as 6000 BC. Barley and wheat cultivation was visible in Mehrgarh by 8000-6000 BC. By the 5000 BC, agricultural communities became widespread in Kashmir and Central Asia (Wood, B. 1996). Cotton was cultivated between the 5000 - 4000 BCE (Stein, 1998). Moreover, the cotton industry of Indus vally was well established and some methods used in cotton fabrication and spinning continued to be practiced till the modern industrialization of India (Wisseman and Williams,1994). The Indian peoples domesticated plant residues - hemp which they used for some applications including making fiber, narcotics, and oil (Krebs and Krebs,2003). Systematic irrigation and water reservoirs were developed by the Indus Valley Civilization, including artificial reservoirs at Girnar, Gujarat dated backs to 3000 BC (Rodda & Ubertini, 2004). Spices like cinnamon and black pepper were grown in India and started as trading shipping spices to the countries of Mediterranean (Adas, 2001).

The concept and practice of utilizing microorganisms to control insect pests had started along with human culture (Candas and Bulla , 2002). Pest control strategies in prehistoric times were mentioned in the writings of ancient Egyptian and Chinese scholars. One story relates the practice by gardeners of several Egyptian kings of maintaining bacterial collections for use against insects that attacked and ravaged the gardens surrounding their houses, societies and tomb chapels. Later, in 300 A.D., maladies of insects, most likely occasioned by bacteria, viruses and fungi were observed. Indeed, Aristotle described in his writings about insect diseases such as foulbrood of the honey bee (*Apis millifera*). Louis Pasteur studied silkworm diseases and differentiated pebrine and flacherie diseases of the silkworm *Bombyx mori.*

1.2 *Bacillus thuringiensis* as biocontrol agent

In 1901, a Japanese scientist named Shigetane Ishiwata isolated a bacterium from dead silkworm larvae while investigating the cause of lost of large numbers of silkworms. He coined name of bacterium – *Bacillus sotto* (Chegasaki, 1915) . Later on, in 1911, Ernst Berliner from Thuringia –state of Germany isolated related Bt strain from dead flour moth larvae and named the organism *Bacillus thuringiensis*. In 1925, he found inclusion bodies alongside the endospore of the bacterium. In mid of 19th, Christopher Hannay referred highly refractive bodies of *Bacillus thuringiensis* " parasporal crystals (Hannay,1953). Thomas Angus promptly demonstrated the insecticidal activity of the inclusion bodies in the same year (Angus, 1953). Hannay together with Fitz-James, in 1955 discovered that the toxic inclusion bodies are composed of protein in nature (Hannay and Fitz-James, 1955). In 1938, France, Europe, the first commercial bioinsecticide based on Bt was produced as Sporine primarily to target to flour moths. In the USA, 1958 commercial production of Bt has been started. By 1961, *Bacillus*

thuringiensis based bioinsecticides were being registered by the US Environmental Protection Agency. Since 1996, the breakthrough of agriculture reported insect resistant transgenic crops, known as Bt crops, have expanded around the world and are proving to be efficient and effective in reducing the use of chemical insecticide (Qaim and Zilberman, 2003.;Kleter et al., 2007). The production of Dipel (*Bt kurstaki*) began was started in 1970 and subsequently proved to be 20–200 fold more potent than other *Bt*-based biopesticides (Beegle & Yamamoto, 1992). Dipel used to control more than 167 lepidopteran pests (Glare & O'Callagham, 2000).The current global market for pesticides (herbicides, insecticides, fungicides, nematicides and fumigants) is valued at $25.3 billion. Farmers have been encouraged to planted *Bt* crops from 1 million hectors in 1996 to 76 million till 2013 (Wei et al., 2015). Latest estimates indicate that more than 50% of the cotton and 40% of the corn planted in the US are genetically engineered to produce Bt insecticidal toxins.

The global planting of biotech crops such as insect resistance Bt cotton and Maize, soyabean, canola, squash have increased 100 fold from 1.7 million hectares in 1996 to 179.7 million hectares in 2015 by up to 17 to 18 million farmers. Farmers benefit estimated at over US$150 billion for the period 1996 to 2015. The accumulated planting of GMO was near to 2.0 billion hectares comprise 1.0 billion hectors of transgenic soybean, 0.6 billion hectares of Bt maize, 0.3 billion hectares of Bt cotton and 0.1 billion hectares of biotech canola. It suggests biotech crops the fastest adopted crop technology in recent times. This impressive adoption rate sounds for itself, regarding its resilience, sustainability and the significant benefits it delivers to both small and large farmers as well as consumers (Clive, 2015). From 1996 to 2015, approximately 18 million farmers, grew biotech crops annually. Remarkably, about 90% or 16.5 million, were planted by small, poor farmers in developing countries. The latest economic data available for the

period 1996 to 2014 indicates that farmers in China gained US$17.5 billion and in India US$18.3 billion. In addition to economic gains, farmers benefited enormously from at least a 50 % reduction in the number of insecticide applications, thereby reducing farmer exposure to insecticides, and importantly contributed to a more sustainable environment and better quality of life. India is the largest producer of cotton in the world. It attributed due to transgenic Bt cotton. Indian farmers planted 11.6 million hectors by around 7.7 million small farmers with 95 % of total planting in 2014. Brookes and Barfoot (2016) reported that India had enhanced farm income from Bt cotton by US$18.3 billion in the year period 2002 to 2014 and US$1.6 billion in 2014 alone.

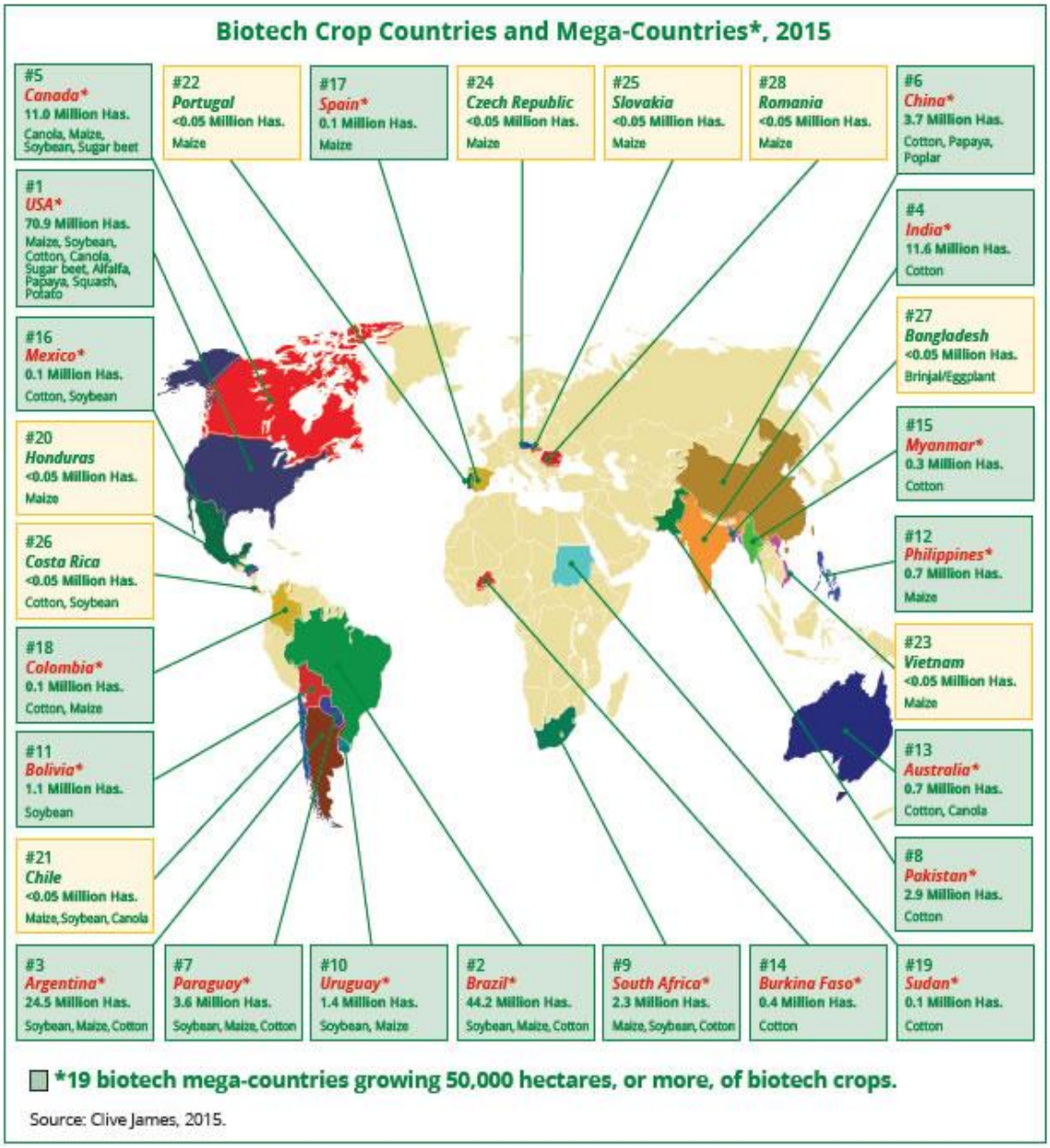


Figure 1.1: Global map of biotech crops cultivation in different countries in 2015 (Clive., 2015)

Table 1.1: Global cultivation of genetically modified plants in 2015 (million hectors) **

(Clive., 2015)

Rank	Country	Area (million hectares)	Biotech Crops
1	USA*	70.9	Maize, soybean, cotton, canola, sugar beet, alfalfa, papaya, squash, potato
2	Brazil*	44.2	Soybean, maize, cotton
3	Argentina*	24.5	Soybean, maize, cotton
4	India*	11.6	Cotton
5	Canada*	11.0	Canola, maize, soybean, sugar beet
6	China*	3.7	Cotton, papaya, poplar
7	Paraguay*	3.6	Soybean, maize, cotton
8	Pakistan*	2.9	Cotton
9	South Africa*	2.3	Maize, soybean, cotton
10	Uruguay*	1.4	Soybean, maize
11	Bolivia*	1.1	Soybean
12	Philippines*	0.7	Maize
13	Australia*	0.7	Cotton, canola
14	Burkina Faso*	0.4	Cotton
15	Myanmar*	0.3	Cotton
16	Mexico*	0.1	Cotton, soybean
17	Spain*	0.1	Maize
18	Colombia*	0.1	Cotton, maize
19	Sudan*	0.1	Cotton
20	Honduras	<0.1	Maize
21	Chile	<0.1	Maize, soybean, canola
22	Portugal	<0.1	Maize
23	Vietnam	<0.1	Maize
24	Czech Republic	<0.1	Maize
25	Slovakia	<0.1	Maize
26	Costa Rica	<0.1	Cotton, soybean
27	Bangladesh	<0.1	Brinjal/Eggplant
28	Romania	<0.1	Maize
	Total	**179.7**	

* 19 biotech mega-countries growing 50,000 hectares, or more, of biotech crops
** Rounded off to the nearest hundred thousand

1.3 Ecology and Diversity of *Bacillus thuringiensis*

Entomopathogenic *Bacillus thuringiensis* is a Gram-positive bacterium found naturally in diverse habitats like dead insects, grains, dust, water, phylloplane and marine sediments etc (Smith and Couche 1991; Meadows et al., 1992; Kaelin et al, 1994; De Lucca et al, 1982). It was also found to be present in diverse environments such as river sedimentary soil (Patel et al, 2011), sedimentary rocks (Baig and Mehnaz 2010), phylloplane and soil environments (Bizzarri and Bishop 2007; Hendriksen et al, 2002), intestine of mammals (Swiecicka et al, 2002), activated sludge (Mizuki et al, 2001), grass foliage (Damgaard et al, 1998), grain storage, mushroom compost (Bernhard et al, 1997) and varied soil and plant types (Martin and Travers 1989).

1.4 Different virulence factors of *Bacillus thuringiensis*

Bacillus thuringiensis is a Gram positive bacteria belong to Bacillaceae family. It produces various biocontrol agents like δ-endotoxins, β-endotoxins, vegetative insecticidal protein (VIP), chitinase, etc. All these agents act as a biological control of various insect pathogens. *Bt* has a wide array of virulence factors to prove itself as a potent entomopathogen. Different strains of *Bt* produce a different kind of virulence factors. Following are virulence factors reported by researchers with the course of time.

1.4.1 Cry toxin

It is the main virulence factors produced by *Bt* during sporulation stage. *Bt* produces the parasporal crystal inclusions which are known as protoxins of Cry protein. Apart from insects of Lepidoptera, Diptera and Coleoptera, Cry toxins showed toxicity to protozoa, arthropods, mites, flatworms, nematodes, human cell lines, etc. (Frankenhuyzen, 2009; Ohba et al., 2009; Chougule and Bonning, 2012). Cry proteins are encoded by *cry* genes which are located on plasmids (Carlton and Gonzalez, 1985). Cry1 to Cry74 type of

different toxins are known till now reported. They possess a wide range of molecular mass from 150 to 13 kDa.

1.4.2 Cyt toxins

They are also produced during sporulation stage of bacteria as protoxins as parasporal inclusions. They are grouped into two families viz Cyt1 and Cyt2 which have molecular masses of 29 - 27 kDa respectively (Schnepf and Crickmore, 1998). Cyt toxins are encoded by *cyt* genes which were found to be located on plasmids. Cyt1Aa (Cohan et al. 2011) and Cyt2B (Akiba et al. 2009) showed that these proteins are single domain, three-layer alpha-beta proteins. Similar to Cry toxins, Cyt toxins gets activated upon removal of small N-terminal and C-terminal portions. However, Cyt toxin does not form oligomeric structures like Cry toxins, but interact directly with membrane lipids in insect gut to form pores (Boonhiang and Ellar 2003). They showed toxicity broad cytolytic activity *in vitro* against a variety of insect and mammalian cells, including erythrocytes, lymphocytes, and fibroblasts (Knowles et al., 1989). However, *in vivo* they are toxic to mosquitoes and black flies.

1.4.3 Vip toxins

VIPs are produced during vegetative phase of growth cycle in contrast to Cry and Cyt toxins which produce during sporulation of *Bacillus thuringiensis* (Palma et al., 2012). They are grouped into Vip1, Vip2, and Vip3 toxins. Vip1 and Vip2 have specificity towards coleopteran insects, whereas Vip3 toxins have lepidopteran specificity (Estruch et al. 1996). The molecular mass of Vip toxins varies from 96 to 50 kDa. Similar to Cry and Cyt, Vip toxins are proteolytically cleaved to an active form which binds to mid-gut membrane receptors and causes pore formation. Vip3A proteins have been shown to act on to target pests by interacting with different receptors than used by Cry proteins (Lee

et al., 2006; Gouffon et al.,2011) which make them alternative to Cry protein for insect resistance management (Ruiz de Escudero et al.,2014).

1.4.4 Exotoxins

Bt strains also produced exotoxin during vegetative phase which is secreted from the cell. It is not protein but a kind of oligosaccharide and shows insecticidal activity. The β-exotoxin or thuringiensin is a heat stable toxin; retained its biocidal activity even at 121 °C for 15 min (Liu et al., 2010). Exotoxin is identical to an ATP analog and can interfere in RNA polymerase activity and reported to have activity towards Lepidoptera, Diptera, Coleoptera, Hymenoptera, Orthoptera and vertebrates (Toledo et al, 1999, McClintock et al., 1995) Another exotoxins called α-exotoxin or PIPLC (phosphatidylinositol-specific phospho-lipase C) is a heat-labile toxin which degrades phospholipids in epithelial membrane of insect gut (Ikezawa 1991).

1.4.5 Proteases and Chitinases

Proteases and Chitinases are also produced as virulence factors by some *Bt strains.* Proteases degrade midgut tissue to allow spores to enter haemocoel and killed insect. Similarly, chitinases caused degradation of the chitinaceous peritrophic membrane of insect for enhancement of pathogenesis. It has reported to such molecules could control pests when applied with synergistically with Cry toxin (Wolfersberge et al. 1996; Chen et al., 2007**).** Other virulence factors produce by *Bacillus thuringiensis* are secret insecticidal protein (Sip), zwittermicin A (ZwA), Mtx-like toxin and Bin-like toxin.

1.5 Classification of insecticidal Cry protein (Cry)

Bacillus thuringiensis is one the most diverse bacterium having a large number of different, pathotypes, serotypes, ribotypes and biochemical types. Thus, classification of *Bt* has remained a challenging task for many years. Classification based on pathotypes

becomes cumbersome due to a large number of toxin coding genes like *cry1 – cry74*, *cyt1A-cyt2C* and *vip1-vip3*. The presence of multiple toxin genes and plasmid exchange by conjugation among *Bt* strains creates more difficulties. Biochemical characterization becomes too cumbersome or requires the use of miniaturized advance systems. Ribotypes classification using advanced molecular has been successful to some extent; however, it is not used regularly by scientists around the world and lacks consistency when applied. Thus, classification based on flagellar H-serotyping becomes significant regarding its uniformity. Currently, more than 400 Bt toxin genes have been cloned and sequenced, including 218 Cry and 28 Vip toxin holotypes (Crickmore et al., 2011)

1.6 Nomenclature of insecticidal Cry/Cyt/Vip toxins and *cry*/*cyt*/*vip* genes of Bt

In 1989, Hofte and Whitely first time revised nomenclature and classification for Cry and Cyt toxins based mainly on insecticidal specificity. Cry proteins being encoded by *cry* genes, and Cyt proteins by *cyt* genes, the *cry* genes were grouped into four classes and several subclasses, and *cyt* genes into one class. Lepidoptera specific *cryI*, diptera and lepidoptera specific *cryII*, coleoptera specific *cryIII* and diptera specific *cryIV* were classified as four classes and diptera specific *cytA* as one class. However, discrepancies were faced with this nomenclature when more crystal protein genes were sequenced and toxicity tested. Cry1Ac and Cry1C toxins showed toxicity to both Lepidoptera and Diptera larvae. Novel toxins did not exhibit the sequence-specificity correlation. Crickmore et al., (1998) therefore proposed a revision of classification based exclusively on amino acid sequence. Cry and Cyt were kept as mnemonic roots, while Roman letters representing primary rank was replaced with Arabic numbers (*cryI* changed to *cry1*) to accommodate the ever growing list of *cry/cyt* genes. New class was assigned only if sequence homology was less than 45%. Secondary and tertiary ranks were represented by a capital and small letters with sequence homology to be less than 78% and 95%

respectively. Additional fourth rank was given by another Arabic number to indicate toxins with identical sequences or slightly different. As of today 21st December 2016, 304 holotype sequences are grouped into 74 classes of *cry* genes and two classes of *cyt* genes. Additionally, *vip* genes encoding Vip proteins are grouped into three classes

Bacillus thuringiensis toxin nomenclature given in (http://www.lifesci.sussex.ac.uk/Home/Neil_Crickmore/Bt).

1.7 Insect toxicity spectrum of *Bacillus thuringiensis* toxins

Exploration of *cry* gene diversity throughout various parts of the world has led to find 74 groups of *cry* genes and characteristic features of each group. The *cry1* gene class is the largest group which has activity mainly towards lepidopteran insects and thus it is more important economically. On the other hand *cry2* gene class though has less number of genes, but is unique in encoding toxicity towards lepidopteran, dipteran and hemipteran insect orders. Each group of toxin showed a specific toxicity for different insect orders such as lepidoptera, diptera, coleoptera, hymenoptera, homoptera, mallophage and acari (Table 1.5) (Frankenhuyzen et al, 2009). In addition, it was reported that *B. thuringiensis* toxins were also able to control some invertabrates such as nemathelminthes, platyhelminthes and sarcomastigorphora (Feitelson, 1993).

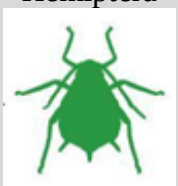
Insect order	Types of Cry proteins
Lepidoptera 	Cry1A-K;Cry2;Cry7B;Cry8D; Cry9A-C,E; Cry15A; Cry22A; Cry26Aa Cry28Aa,Cry32A; Cry51A
Dipteral 	Cry1A-C; Cry2A; Cry4A-B; Cry10; Cry11A-B; Cry16A; Cry17Aa ; Cry19A-B; Cry20A; Cry24Aa ;Cry24C; Cry26Aa, Cry27A; Cry29Aa ; Cry30Aa,C ry32B-D; Cry39A; Cry40Aa-Ba ;Cry44A;Cry47A; Cry48A; Cry49A; Cry50Aa; Vip3A(a-g), Vip3B(a-b);Cyt1A-B;Cyt2A-B
Coleopteran 	Cry1B; Cry3A-C; Cry7A; Cry8A-G; Cry9D; Cry14A; Cry18A; Cry22A-B; Cry23; Cry34A-B; Cry35A-B; Cry36A; Cry37A; Cry43A-B; Cry43A-B; Cry55A; Vip1A(a-b), Vip1B(a-b), Vip1Ca, Vip1Da, Vip2A(a-d), Vip2B(a-b) Cyt1A;Cyt2C
Rabhtidita 	Cry5A-B; Cry6A-B; Cry12A; Cry13A; Cry14A; Cry21A; Cry55A
Hymenoptera 	Cry3A; Cry5A; Cry22A
Hemiptera 	Cry2A, Cry3A; Cry11A
Human cancer cells 	Cry31A; Cry41A; Cry42A; Cry45A; Cry46A

Table 1.2: Specificity of different Cry proteins to insects

1.8.1 Advantages of *Bt* being Biocontrol agent

Bacillus thuringiensis offers numerous advantages over chemical pesticides and other biopesticides

- There is no waiting period before application to the field.
- Different strains of *Bt* are class-specific. Thus, non-target insects are not harmed.
- *Bt* remain non-toxic to humans, mammals, pets and others . This high margin of safety recommends its use on food crops or in other sensitive sites where chemical pesticide use can cause adverse effects.
- The insects that ingested crystal proteins and later died. These cadavers of insects were not dangerous to birds or other animals that may feed on the dead insects.
- *Bt* spore-crystal is not known to cause injury to plants on which it has been applied and is not considered harmful to the environment.
- There have been a few cases of *Bt* resistant reported to date. However, pesticide rotation and cultural control methods should be used to retardation or elimination of the possibility of resistance development. Reapplying on any one pesticide can lead to the buildup of resistance in the pest population. Overuse must be avoided.
- *Bt* spore-crystal of a particular strain can be produced easily on submerged or solid media fermentation conditions – a key factor in its successful development as a biopesticides.

1.8.2 Disadvantages of Bt

- It lacks transmission and spreading facilities. It must be applied like a chemical insecticide.
- It is only effective against less than third instar of lepidoptera, diptera, and coleoptera larvae feeding on exposed plant surfaces. This limits its usefulness against insect pests that borrow into the plants or plant parts. Examples include codling moth and corn earth worm which are susceptible to *Bt* but rarely have an opportunity to eat it in field use.
- Insecticidal property of Bt is short residual effect. So, frequent applications are required compared with chemical insecticides. *Bt* spores- crystals are susceptible to degradation by sunlight and most \formulations persist on foliage less than a week following application..
- *Bt* based products tend to have a shorter shelf life than other insecticides. Manufacturers generally indicate reduced effectiveness after two or three years of storage, with liquid formulations being more perishable than dry formulations Shelf life is greatest when storage conditions are cool, dry, and out of direct sunlight.

1.9 Three domain structure of Cry protein

Insecticidal Cry toxin belongs to the three-domain Cry toxin family and exhibited clear differences in their amino acid sequences. However, all proteins share remarkably similar and conserved their three-domain structure.

Domain I has function in pore formation in epithelial membrane of insect gut. Domain I remain the most conserved among Cry toxins. It is constituted by a seven α-helixes. Mutation in this domain particular to active site lead to complete loss of toxicity toward

insect larvae. Protein engineering of Cry1Ab was used to determine the ability of this α-helix. It was found that mutation at L157C and S176C of α-helix 5 of Cry1Ab were important for ion-transport in membrane (Alzate et al. 2009). While other mutation study reported to the change in susceptibility of two different hosts. Site directed mutation at V171C showed important differences in larval susceptibilities between *M. sexta* and *L. Dispar*. The mutated toxin showed a 25-fold increase in toxicity to L. Dispar (Alzate et al.2010).

Domain II has role mainly in specificity determination of insect larvae. It has most variable region which is consist of three sets of antiparallel β-sheets, each terminating with a loop. The beta sheets were packed around a central hydrophobic core forming a so-called beta-prism structure (Grochulski et al. 1995). Loop regions of domain II, which are involved in receptor binding and toxin specificity (Pigott and Ellar 2007; Rajamohan et al. 1995, Schnepf et al. 1998).The hydrophobicity of some residues of loop were very important in regulating the molecular interactions between the domain and receptors on the target membranes as well as regulation of the translocation process of the toxins in membrane.

Domain III is participating in structural stability of toxin. However, it has been reported that domain III has role in specificity as well as in toxicity against insect larvae. Domain III is a sandwich of two antiparallel β-sheets that form a "jelly-roll" topology. Rearchers reported site-directed mutagenesis and analysis of truncated form provide strong evidence for the involvement of Domains III has role in receptor binding and insecticidal activity. Aronson et al., (2012) reported that single Ala substitutions of two Ser residues at positions 503 and 504 in the Cry1Ac toxin decreased binding affinity of the toxin and reduced toxicity to the tobacco hornworm. The specificity of the amino acid residue Thr524 located in the β-16-β17 loop of domain III for receptor-binding interactions has

been used to increase the activity of Cry1Ac towards Lepidopteran insects (Shan et al. 2010).

The three dimensional crystal structure of Cry-proteins have been determined such as Cry1Aa (Grochulski et al., 1995), Cry2Aa (Morse et al., 2001), Cry3Bb (Galitsky et al., 2001), Cry3Aa (Li et al., 1991), Cry4Aa (Boonserm et al., 2006) ,Cry4Ba (Boonserm et al., 2005), Cry8Ea1 (Guo et al., 2009), activated Cyt2Ba (Cohen et al., 2008), unprocessed Cyt2Aa (Li et al., 1996). All these structures have three domains structure. Although the multiple sequence alignment of these toxins is low, the overall structural topology of the three structural domains is near to similar. Lopez et al., (2013) determined structure alignments between Cry1Aa and other Cry toxin structures by using the FATCAT algorithm (Figure 1.3).

1.10 Non three dimensional structure of Cry toxins

Some toxins exhibited low level of primary sequence identity to Cry toxin but showed no similarity to three domains like Cry toxins also considered in Cry protein nomenclature. They classified under distinct primary rankings in the nomenclature. Toxins namely Cry15, Cry23, Cry33, Cry38, Cry45, Cry51, Cry60 and Cry64 fall in ETX_MTX2 family of Clostridium epsilon toxin. These toxins are identical to aerolysin, a pore-forming toxin produced by the Gram-negative bacterium (Gonzalez et al., 2008; Knapp et al. 2010). Moreover, other toxins belong to the Toxin_10 family of proteins like BinA, BinB, Cry35, Cry36 and Cry49 from Bt. Cry22 is reported as cadherin-like domains and a C-terminal region which exhibit structural similarities to domain III of the ordinary Cry toxin (De Maagd et al., 2003). Cry35 has a beta trefoil N-terminal domain containing QxW motifs similar to those found in carbohydrate binding domains in proteins, such as

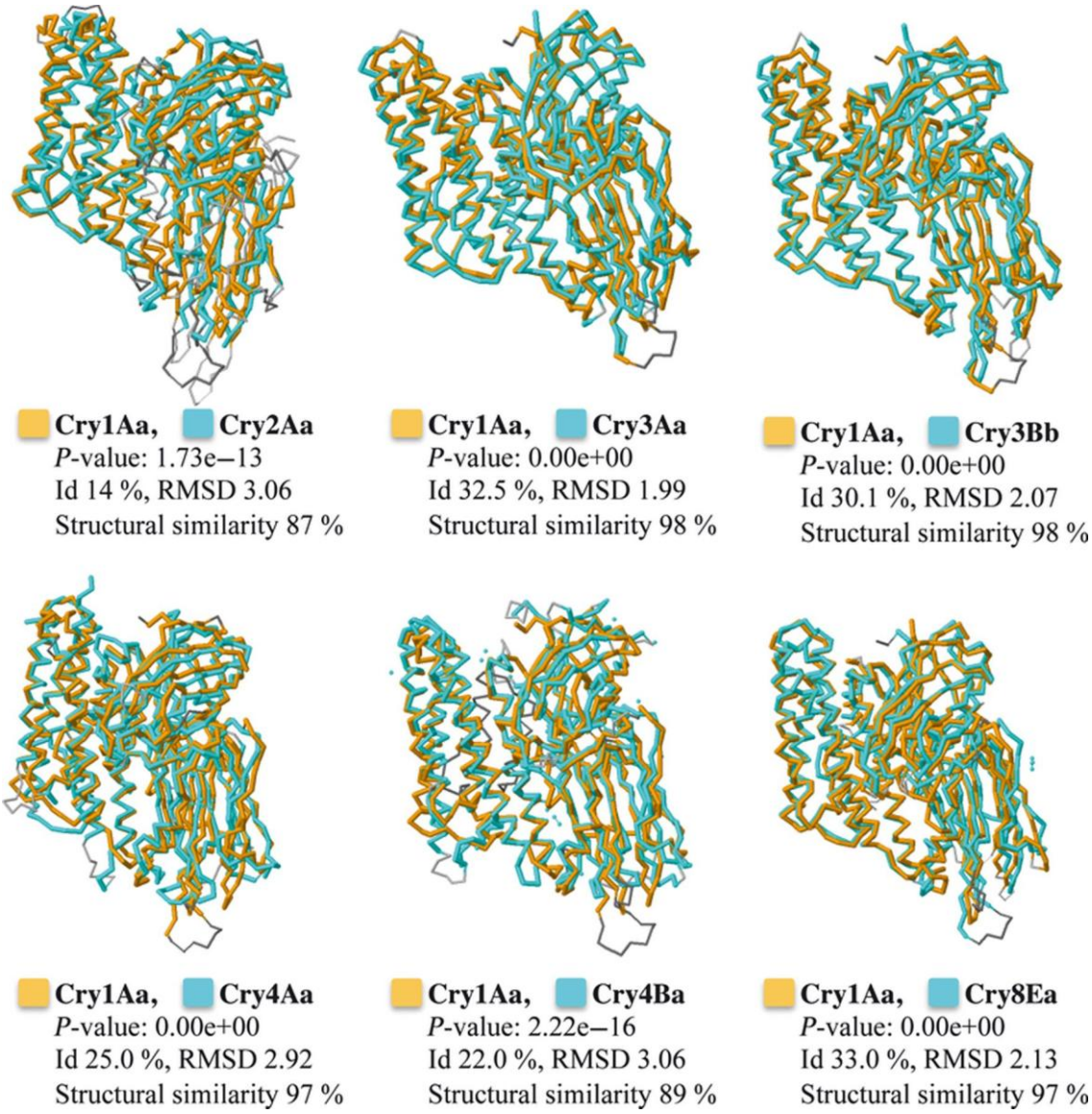


Figure 1.3: Structural similarity of Cry1Aa and other Cry toxins (Lopez et al., 2013)

ricin and Mtx1 family (Kelker et al., 2014). Other toxin Cry34 from aerolysin family, its published structure shows a single domain protein with a beta-sandwich conformation and a hydrophobic core (Figure 1.4). Since no obvious homology at the level of their amino acid sequences, the Cry34/Cry35 pair showed remarkable structural similarity to other binary toxins Cry23/Cry37 (de Maagd et al., 2003).

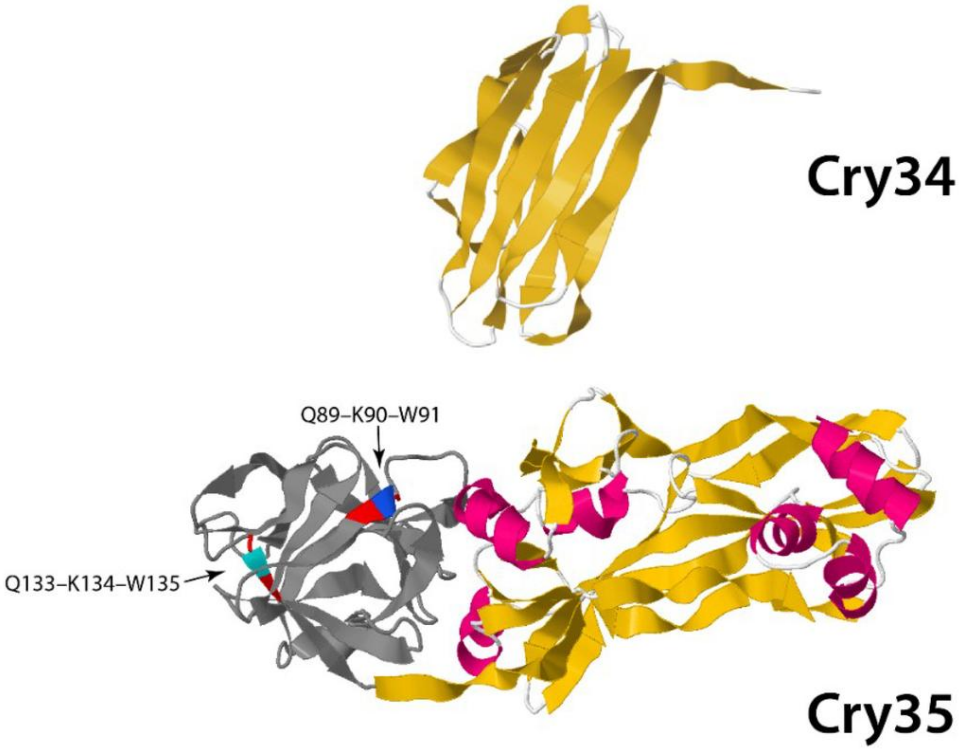


Figure 1.4: Three-dimensional structure of binary Cry34/Cry35 toxin (de Maagd et al., 2003).

1.11 Mechanisms of Cry toxin action

The protoxin form of Cry toxin ingested by susceptible larvae pests solubilised in presence of alkaline condition of insect gut (Hofmann et al.,1988). It convert into ~ 70 - 65 KDa active form after proteolytic cleavage of protoxin (Choma et al.,1991). The activated Cry toxin through a complex binding process with different receptors present on surface of epithelial membrane. First binding interaction of activated Cry toxin with low affinity and reversible manner to ALP and APN receptors till reached optimum concentration on surface of microvilli membrane. In lepidopteran insects four different receptors have been described for Cry1A toxins which are cadherin-like protein (CADR), glycosylphosphatidyl-inositol (GPI)-anchored aminopeptidase-N (APN), GPI-anchored alkaline phosphatase (ALP) and glycoconjugate (Vadlamudi et al, 1995; Knight et al., 1994; Jurat-Fuentes et al, 2004; Valaitis et al, 2001). Then, exposed loop 2, 3 and α -8 of

domain II of toxin bind to CAD receptor with high affinity which induced proteolytic cleavage of the N terminal end including helix α -1 of domain I. Oligomerization of activated Cry toxin monomers occurred before insertion into membrane. Oligomeric structure bind with higher affinity and irreversible to ALP and APN receptors compared to initial binding. Oiligomeric structure complex of toxin could inserted into theof membrane and formed pores. Ultimately, insect larvae die due to septmia (Figure 1.5).

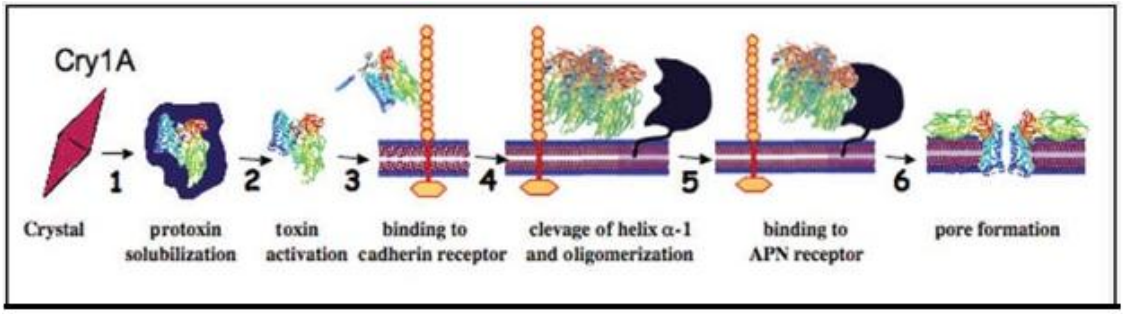


Figure 1.5 : Schematic representation of mode of action of Cry toxin (Bravo et al., 2004)

1.12 Mechanisms of resistance to Cry toxin in different insects

Resistance to Cry toxin could occur by sequestering any step of Cry toxin mode of action in gut of insect larvae (Figure 1.6). The most common mechanism of toxin resistance is the reduction in toxin binding to receptors at midgut cells of different resistant insect species. Mutations in Cry toxin receptors such as CAD, ALP, APN or in the ABCC2 transporter (Jurat., 2004). In fact, resistant insect populations selected in laboratory conditions have shown that resistance can be developed by different mechanisms such as altered activation of Cry toxins by midgut proteases (Killer and Sneh 1996; Oppert et al., 1997; Li et al., 2004), sequestering the toxin by esterases (Gunning et al.,2005), by inducing an elevated immune response (Rahman et al., 2004), as well as alteration resulting in reduced binding to insect gut membranes

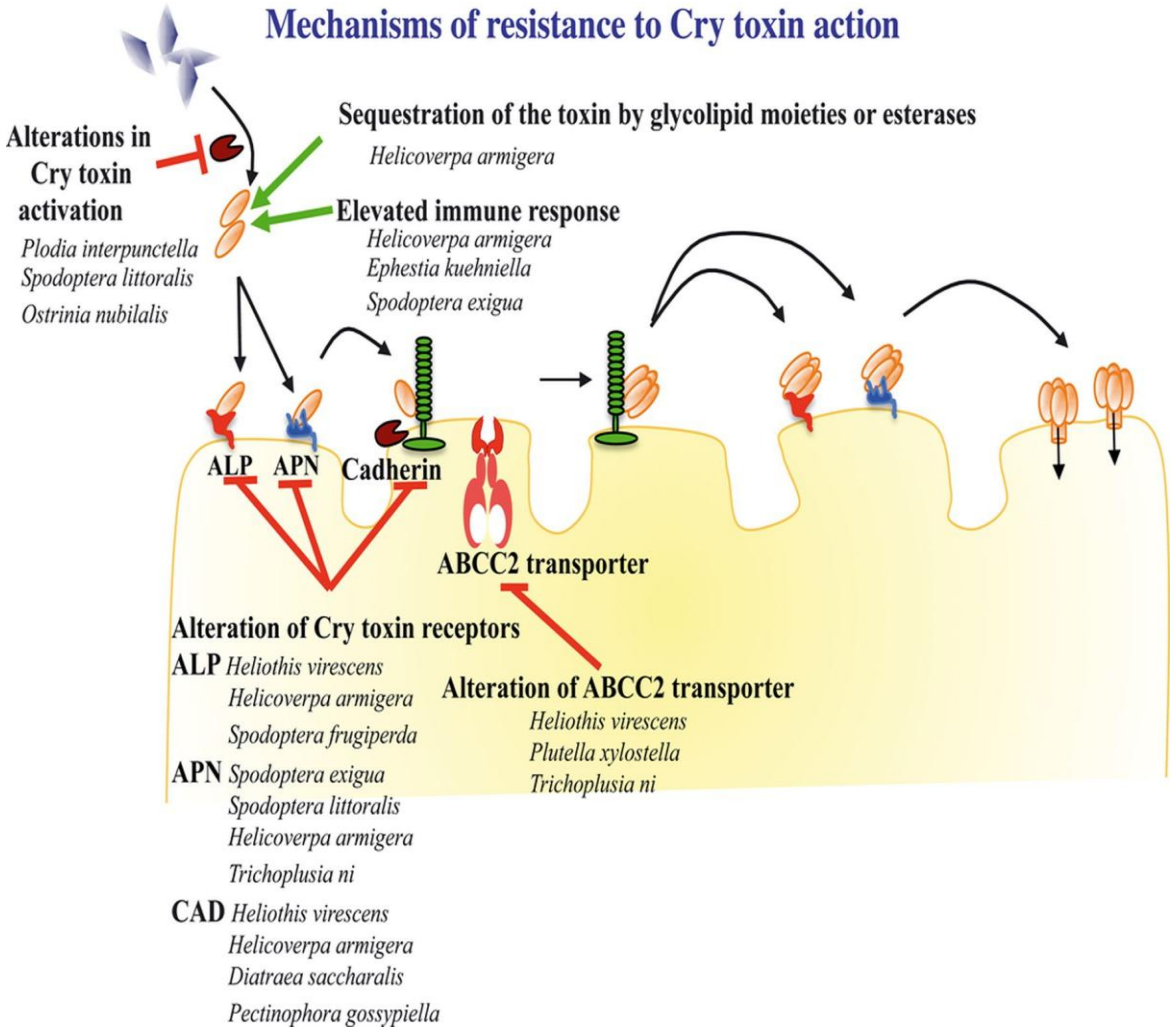


Figure 1.6: Schematic representation of the different mechanisms of resistance to Cry toxin reported in lepidopteran insects. (Pardo Lopez et al., 2013)

Toxin binding to BBMVs has been reported to be linked to mutation in toxin receptors (Ferre and Rie., 2002). It has been reported that some Cry-tolerant insects such as *Helicoverpa armigera* released lipid particles into the gut lumen. The glycolipids that are present in these lipid particles bind Cry1Aa and Cry2Ab toxins resulted in toxin sequestration in the gut lumen, thereby affecting the interaction of the toxins with specific receptors on midgut cells, acting as a trap for mature toxins (Ma *et al*., 2012). In addition, it was reported that sequestration of Cry1Ac in the 275 fold tolerant *H. armigera* strain is

also due to binding of the toxin to esterases (Gunning *et al*., 2005). The role of elevated immune responses that resulted in a 16-fold greater tolerance to Cry toxins in *Ephestia kuehniella* or 12-fold resistance in *H. armigera* larvae has been documented. In these insects the rate of melanization reactions was increased resulted in tolerance to 3d-Cry toxin intoxication (Rahman *et al*., 2004; Ma *et al*., 2005). In *Spodoptera exigua*, an elevated immune response was described by analyzing differential gene expression in a 100-fold more resistant population to the *Bt*-formulated product Xentari (Hernandez-Martinez *et al*., 2010).These reports indicated that alterations in insect gut physiology could result in resistance to *Bt* toxins.

1.13 Bt toxins as insect resistance transgenic plants

The first transgenic plants to express Bt toxins were tobacco and tomato plants (Van Frankenhuyzen., 1993). Transgenic Bt crops are especially helpful against pests that attack parts of the crops that are usually not well-protected by conventional insecticide application. The first Bt plant was Bt corn registered with the United States EPA in 1995. Today, major Bt transgenic crops also include corn, cotton, potatoes, and rice.

Generally, these transgenic Bt crops have modified insecticidal proteins with molecular weights between 88 kDa and 65 kDa (Hofte and Whiteley., 1989) and are known to be lethal against coleopteran and lepidopteron insects. In 2015, Indian farmer planted Bt cotton (Ballguard-II) in 11.6 million hectors covering 95% of total cotton (Malthankar and Gujar., 2016). Table 1.3 presented commercial available Bt crops along with their tradenames.

Table 1.3: Commercial available transgenic Bt crops (Castagnola and Fuentes. 2012)

Company	Crop	Name of hybrid	Trade name	Bt gene	Pest*
Syngenta	Maize	Bt11	Agrisure CB/LL, Agrisure GT/ CB/LL	Cry1Ab	ECB
		MIR604	Agrisure RW, Agrisure RW/ GT	Cry3Aa	WCR
		Bt11, MIR604	Agrisure 3000GT, Agrisure CB/ LL/RW	Cry1Ab,Cry3Aa	ECB WCR
		Bt11, MIR162	Agrisure Viptera 3110	Cry1Ab, Vip3Aa20	ECB,FAW, CEW,BCW,WBC
		Bt11, MIR604, MIR162	Agrisure Viptera 3111	Cry1Ab, Cry3Aa Vip3Aa20	ECB,WCR,FAW CEW,BCW,WBC
	Cotton	COT102, COT67B	VipCot	Vip3Aa19, Cry1Ab	CBW, TBW, PBW, FAW, BAW, SBL, CL, CLP
Monsanto	Maize	MON810	YieldGard Corn Borer	Cry1Ab	ECB
		MON863	YieldGard RW	Cry3Bb1	CRW
		MON810, MON863	YieldGard VT Triple, YieldGard Plus	Cry1Ab, Cry3Bb1	ECB, CRW
		MON89034	Genuity VT Double PR	Cry1A.105/ Cry2Ab	CEW,ECB,FAW
		MON89034, MON88017	Genuity VT Triple PRO	Cry1A.105/ Cry2Ab, Cry3Bb1	CEW,ECB,FAW CRW
		MON89034, TC1507, MON88017, DAS-59122-7	Genuity SmartStax	Cry1A.105/ Cry2Ab2, Cry1Fa2, Cry3Bb1, Cry34/35Ab1	BCW, CEW, CRW, ECB, FAW, WBC, SCB, SWCB, SCSB, CEW, SCB, WBC, WCR
	Cotton	MON531	Genuity Bollgard, Ingard	Cry1Ac	PBW, TBW
		MON15985	Genuity Bollguard II	Cry1Ac/ Cry2Ab2	PBW, TBW,CBW
Pioneer (DuPont) and Dow Agrosciences	Maize	DAS-06275-8		Cry1F	BCW, ECB, FAW, WBC, SWCB, CEW
		TC1507	Herculex I	Cry1F	BCW, ECB, FAW, WBC, SWCB, CEW
		MON810, TC1507, MON810	Optimum Intrasect	Cry1Ab Cry1F, Cry1Ab	ECB ECB, WBC, BCW, FAW
		DAS-59122-7	Herculex RW, Optimum AcreMax RW	Cry34/35Ab1	WCR
		DAS-59122-7, TC1507	Optimum AcreMax 1, Herculex Xtra	Cry34/35Ab1, Cry1F	WCR,BEC, ECB, FAW, WBC
	Cotton	3006-210-24, 281-24-236	WideStrike	Cry1Ac, Cry1F	CBW, PBW, TBW, ECB, SBL, BAW, FAW

*Common insect name abbreviations are: ECB- European corn borer; WCR- Western corn rootworm; FAW- Fall armyworm; CEW- Corn earworm; BCW- Black cutworm; WBC- Western bean cutworm; BEC- Bean cutworm; CBW- Cotton bollworm; TBW- Tobacco budworm; PBW- Pink bollworm; BAW- Beet armyworm; SBL- Soybean looper; CL- Cabbage looper; Cotton leaf perforator; SCB- Sugar cane borer; SWCB- Southwestern corn borer; SCSB- Southern cornstalk borer; SPB- Spotted bollworm.

Transgenic Bt crops are prepared through transformation techniques a modified Ti plasmid system in *Agrobacterium tumefaciens*, microinjection of DNA into cultured cells direct gene transfer including (polyethlyne glycol) PEG-induced DNA uptake, microprojectile and electroporation (Babu et al,, 2003) The advantages over Bt crops plantation apart from high production of yield include reduction in pesticides usage. It was reported that pesticides for the accumulative years 1996 to 2011 was estimated at 473 million kg of active ingredient, a saving of 8.9% in pesticides In 2011 alone, there was a reduction of 37 million kg, equivalent to a saving of 8.5% in pesticides (Jindal., 2013). Other benefits lessen reduction in harmful effects of pesticides on non-target organisms, and reduced amounts of pesticide residues in food and food products (Brookes and Barfoot., 2013).

In 2014, majorly a total four major genetically modified plants viz. soyabeen, cotton, maize and canola were planted in the world. Transgenic Bt cotton accounted for 75% of the total plantations. In India, insect resistance Bt cotton was commercialized by 2002, its planting was increased up to 11.7 million hector till 2014 with share 92% of the total cotton plantation. Bt cotton grower farmers income has been increased by $ 3.94 billion in 2014 from $ 44.8 billion in 1996. In same year 2014, the largest income obtained from transgenic Bt maize is $5.3 billion in developing countries which was equivalent to 3.2% of $163 billion value of global maize crop production. Since 1996, Bt maize crop worth of $41.4 billion created an income to global maize farmer (Brookes and Barfoot 2016).

1.14 Resistance development in insect against transgenic Bt crops

Insect resistance to Bt plants have been reported due to continues pressure of toxin to insect pests. In lab, resistance is associated with alterations in toxin binding to mid gut receptors, which was inherited as a single autosomal recessive gene (Ferre and van Rie.,

2002). In China, *H .armigers* has developed resistance against Cry1Ac expressing Bt crop (Liu et al., 2010). At field level, first reports of Bt resistance under field conditions observed in the *Plutella xylostella* and *Diamondback moth* insect pests, as well as in green house populations of *Trichoplusia ni*. Then, other insect species such as *Spodoptera frugiperda* to Bt corn containing Cry1F in Puerto Ricoin, *Helicoverpa zea* to Cry1Ac cotton in the south-eastern United States, *Busseola fusca* to Bt corn producing Cry1Ab in South Africa.Currently, lepidopteron pest pink ball worm (*Pectinophora gossypiella*) established resistance against transgenic Cry1Ac expressing cotton plant in India (Dhura and Gujar., 2009), United states (Tabashnik et al., 2010) and China (Wan et al., 2012).

1.15 Insect resistance management against Cry toxin

Successful commercialization of Bt transgenic crops is depend upon resistance management practice. Monitoring pest densities and evaluation of economic injury levels so that insecticides applied only when necessary. There is a unique management require for each Bt crop depended pest (Shelton et al., 2002). To delaying resistance, recommendations include usage of high dosage of toxin which can cause minimum chance of survival of the resistant heterozygotes, planting of non Bt transgenic to sustain the homozygous susceptible insect population (Tang et al.,1997). Refuges of non Bt plants can delay resistance by providing susceptible individuals to mate with resistant insects and Bt selecting against resistance. The limitation of this strategy is gene flow between insect population feeding on Bt crops and non Bt plants (Bourguest et al., 2000). Other molecular strategies, expression of tissue specific *cry* genes or expression induced via a chemical spray such as salicylic acid, *cry* gene pyramiding, over expression in chloroplast, novel hybrid genes or synthetic modification of hybrid genes for broader range of insecticidal activity (Christov et al.1999; Kota et al., 1999; De Cosa et al., 2001).

1.16 Microbial formulation for sustainable agriculture

Microbial agents when applied in the field perceive many problems with respect to unfavourable environmental conditions. They have to expose variable temperatures, inadequate pH, draught, and adsorption by soil particles, or washing-off by rain, competition of better-adapted by indigenous microorganisms (Yang et al., 2006; Guo et al., 2012). Formulation is a process to preparation of active ingredients produced by microorganisms mixed with other ingredients which make active ingredients long effective upon application. The composition and characteristic of formulation vary by each formulation. Generally, they depend on technical factors such as aerial or land based formulation application, rheology of inert materials like particle size, viscosity and density, kind of application in Kg h^{-1} or L h^{-1}. While, in nontechnical parameters include insect type, habitat, life cycle, oral or contact based mode of action, insect resistance towards active ingredient. Generally, formulation can be classified dry solid formulation like dusts, powders, granules and briquette, wettable liquid formulation include suspension, water in oil emulsion and oil in water emulsion

1.16.1 Dry solid products

1.16.1.1 Dusts

Dusts were formulated by the absorption or adsorption of an active ingredients on to fine solid inert material like talc, clay or chalk (Couch et al.,1981). The particle size would be in range of 50 -100 µM. *Bacillus thuringiensis* based dusts have been widely used to control surface dwelling lepidopteran pests such as cabbage looper, semi looper (Lynch and Robinson., 1980). Bt dusts have been successful in biocontrol because uniform coverage of under leaf area, protection from sunlight. However, fall down of large size particle proved to limit their applications by farmers (Behle et al., 1996; Ifoulis et al., 2004)

1.16.1.2 Granules

They are discrete mass in range of 5-10 mm^3 prepared by carriers material like starch, clay minerals, dry fertilizers and plant residues (Green, 2000). Granules prepared by mixing 5-20 % of active ingredients or microorganisms to liquid carriers than extruded through a granulation die. There are three types of main granules used such as exterior granules - active ingredient are attached to outer surface of a carrier by a sticker; exterior granules - it prepared by without sticker; incorporated granules – all institutes were mixed into a paste to uniform matrix subsequently sieved to desired size. Bt based granules like casein (Behle et al.,1996); gluten (Behle et al.,1997); cotton seed flour, gelatin or acacia gum (Maldonado et al., 2002) gelatinized corn starch (Dunkle and Shasha 1988; Tamez et al., 1996).

1.16.1.3 Briquettes

Briquettes are large blocks in size of 100 - 250 µM with inert materials like organic polymers like polyvinal alcohol which can allow flotation of briquetters and sustain release of toxin till several months (Aly et al., 1987), Bti formulation widely prepared as abriquettes to control mosquitoes up to two months in single application (Sulaimam et al.1990; Skovmand and Sanogo, 1999).

1.16.1.4 Wettable powders (WP)

It made up of by 50-80% technical powder, 15-45 % filler, 1-10 % dispersant and 3-5 % surfactant. Silica used to as filler to resist cake formation and friability during grinding. Dispersant must added in order to retain suspension in dispersion. WP has longer shelf life, easily miscibility with water and easily in all dry formulation (Burges, 2012) Bt WP products of Valent biosciences and Novartis corporation reported to successful in biopesticides market.

1.16.2 Liquid suspension

1.16.2.1 Suspension concentrates (Flowables)

They are particulates suspension in liquid having 10-40% active ingredient, 1-5 % dispersant, 1-3 % suspender, 3-8 % surfactant, and 35- 65% carrier liquid. Tween and Triton series were widely used as surfactant act as wetting agent and spreader (Tamez et al. 1999) Dispersant can prevent settling of liquid increase shelf life. It reported to addition of sorbitol as a dispersing agent could act as anti freeze, anti evaporator, enhanced density of Bt formulation resulting reducing loading cost (Tamez et al., 2000)

1.16.2.2 Emulsion

It composed of liquid droplets dispersed in another immiscible liquid. It has advantage over suspension concentrates that do not counter sedimentation problem. In water in oil emulsion, oil is external phase losses due to evaporation and spray drift is negligible (Aven and Hasui, 2002; Gasic and Tanovic, 2015)

1.16.2.3 Encapsulation

They are liquid suspension having powder and granules of active ingredients coated by polymers. Microbial biocontrol agents were encapsulated in a capsule made up of polymer such as gelation, starch, cellulose (Chen et al., 2013) Microcapsules have been highly used with small size to prepare fungal biopesticides formulation (Shah and Pell 2003). The advantages using encapsulation based formulation are cell viability retained in finally prepared formulated product till three to four months (Szczech and Maciorowski , 2016).

1.17 Bt spore-crystal formulation for biocontrol of pests

Despite the remarkable characteristics of *Bacillus thuringiensis* as a pest control of different members of insect classes, the short persistence of Bt based biocontrol after field application has become a limitation factor in success. Viability of spores and insecticidal activity of crystal proteins rapidly deteriorate in field conditions (Leong et al., 1980; Dulmage and Aizawa, 1982). Inactivation of spores and δ endotoxins caused by sun light and UV light which consequently depletion in their efficacy to control pests. Bt is accounted to be highly sensitive to UV radiation (Du and Nickerson, 1996; Cucchi and Rivas , 1998). Griego and Spence, (1978) reported that UV radiation in spectrum UV-B (280-400 nm) and UV-C (100-280 nm) mainly responsible for inactivation of Cry toxin toxicity to insect pest. Thus, it need to be increase tolerance of spores and Cry toxins to environmental stresses such as UV radiation, rain and temperature in order to maintain insecticidal activity.

To address this problem, several protective methods have been reported for retained efficacy and performance of spores and δ endotoxins. Encapsulated Bt formulation prepared by maize flour proved to be UV radiation protection and improvement in stickiness in simulated rainfall. Similarly, bioencapsulated formulation prepared by rice flour extended period of mortality against *Choristoneura rosaceana* compared to commercial Dipel and wet table powder formulation (Cote et al., 2001). Ice pellets based low economic formulation of *Bacillus thuringiensis israelesis* used to control mosquitoes too (Beacker. 2003). Also, using of polyethylene glycol (PEG) and ethylene glycol in encapsulation of spore-crystal of Bti provided anti freezing properties (Lew et al., 1997)

Table 1.4 Commercial registered Bt formulation for commercial application (Sansinenea ,2012)

Bt subspecies	Product name	Company	Target insect
	Dipel	Abbott Labs. (Now Valent Bioscience Co.)	Lepidopteran
	Biobit, Foray	Valent Bioscience Co. and Novo Nordisk	Lepidopteran
	Condor, Cutlass, Crymax, Lepinox	Ecogen Inc.	Lepidopteran
	Javelin, Thuricide	Sandoz Agro, Inc.	Lepidopteran
kurstaki	Bactospeine, Futura	Solvay & Cie/duphar B.V.	Lepidopteran
	Bernan Bt	Bactec	Lepidopteran
	Bactis	Compagnia di Recerca chim. CRC	Lepidopteran
	Biospor	Farbwerbe-Hoechst	Lepidopteran
	Larvo-Bt	Knoll Bioproducts	Lepidopteran
	Bt	Korea Explosives	Lepidopteran
	Sporoine	LIBEC	Lepidopteran
	M-peril	Mycogen	Lepidopteran
	SOK	Nor-Am Chemical	Lepidopteran
	Plantibac	Procida	Lepidopteran
	Baturad, Nubilacid	Radonja	Lepidopteran
	Able, delfin, CoStar, Steward, Vault	Thermo Trilogy Corporation	Lepidopteran
	Bactur	Thompsoni Hayward Co	Lepidopteran
	Toaro, Toaro, Ct	Towagosei Chem	Lepidopteran
	Agree[a]	Sandoz Agro Inc.	Lepidopteran
aizawai	Florbac, Xentari	Valent Bioscience Co.	Lepidopteran
	Tobaggi	Dongbu Hannong Chemicals	Lepidopteran
	Solbichae	Gree Biotech Co.	Lepidopteran
	Selectgyn	Kyowa-Hakko Kogyo Co.	Lepidopteran
	Gnatrol	Valent Bioscience Co.	Dipteran
israelensis	Bactimos, VectoBac,		
	Teknar	Valent Bioscience Co.	Dipteran
	Skeetal	Novo Nordisk	Dipteran
	Baktokulicid	VPO Biopreparat	Dipteran
morrisoni or	Moskitur	JZD Slusovice	Dipteran
	M-One, M-Trak	Mycogen	Coleopteran
	Trident, TridentII	Sandoz Agro Inc.	Coleopteran
tenebrionis	Di Terra	Valent Bioscience Co.	Coleopteran
	Novodor	Novo Nordisk	Coleopteran
	Foil[b]	Ecogen Inc.	Lepidopteran Coleopteran
galleriae	Entobaktirin	Glavmikro-Bioprom	Lepidopteran
	Spicturin	Tuticorin Alcali Chemicals and Fertilisers Limited	Lepidopteran
thuringiensis	Bathurin	Chamapol-Biokrna	Lepidopteran
	Muscabac	Farmos	Lepidopteran
	Insektin	Glavmikro-Bioprom	Lepidopteran
dendrolimus	Bacillex	Shionogi Co.	Lepidopteran
	DendroBacillin	Glavmikro-Bioprom	Lepidopteran

[a] Combination of subsp. *kurstaki* and *aizawai*
[b] Combination of subsp. *kurstaki* and *tenebrionis*

1.17.1 Criteria for ideal Bt spore-crystal formulation

Following some criteria must be satisfied by all formulations to be commercially successful of *Bacillus thuringiensis* as per suggested by (Couch, 2000; Brar et al., 2006)

- Insecticidal activity of Bt spore-crystal must be maintained during the formulation process.
- Formulation would be prepare such a way so that the active ingredient is moderately resistant to wash off and UV light in the applied field.
- The formulation must contain excellent stability in environmental conditions.
- The formulations must be easily resuspended and applied under field conditions.
- The formulations must be designed to ensure adequate distribution of the active ingredient and to enhance deposition properties to plant surface. This maximises the presence of the active ingredient on the target application site.

1.18 Nanotechnology in crop protection

The huge challenges of agricultural researchers are to accomplish food requirement of increasing human population of the world. The human population was 1.7 billion in the year 1900; expected to be 9.7 billion by 2050 and 11 billion at the end of 2100. Currently, in the world, 2 billion people are malnourished, and 870 million people are currently chronically hungry (Clive, 2015). Large numbers of insect pests, crop diseases and weed could lead to accounting total 40 % lost of total production (Kashyap et al., 2015). To manage these losses, farmers always promoted to apply agrochemical which resulted in deterioration of soil quality, degradation of the ecosystem, biomagnification, environmental pollution, resistance development in crop pests. Thus, there is an urgent need to change of application of agrochemicals in the field (Chena et al., 2011). The changes include rapid detection of pathogens, soil health promotion by degradation of agrochemical etc (Handford et al., 2014).

Nanotechnology is emerging branch of science which has broad and different applications in agriculture. It includes nanoparticles with the property of defeating plant pathogens, nanoencapsulated minimizing nutrition losses in fertilization, improving crop production through water and nutrient management, delivery of pesticides by nanodevices to enhancement in efficacy of pesticides at a lower rate. Main goals of nanoencapsulation in pesticides application represents in figure 1.7

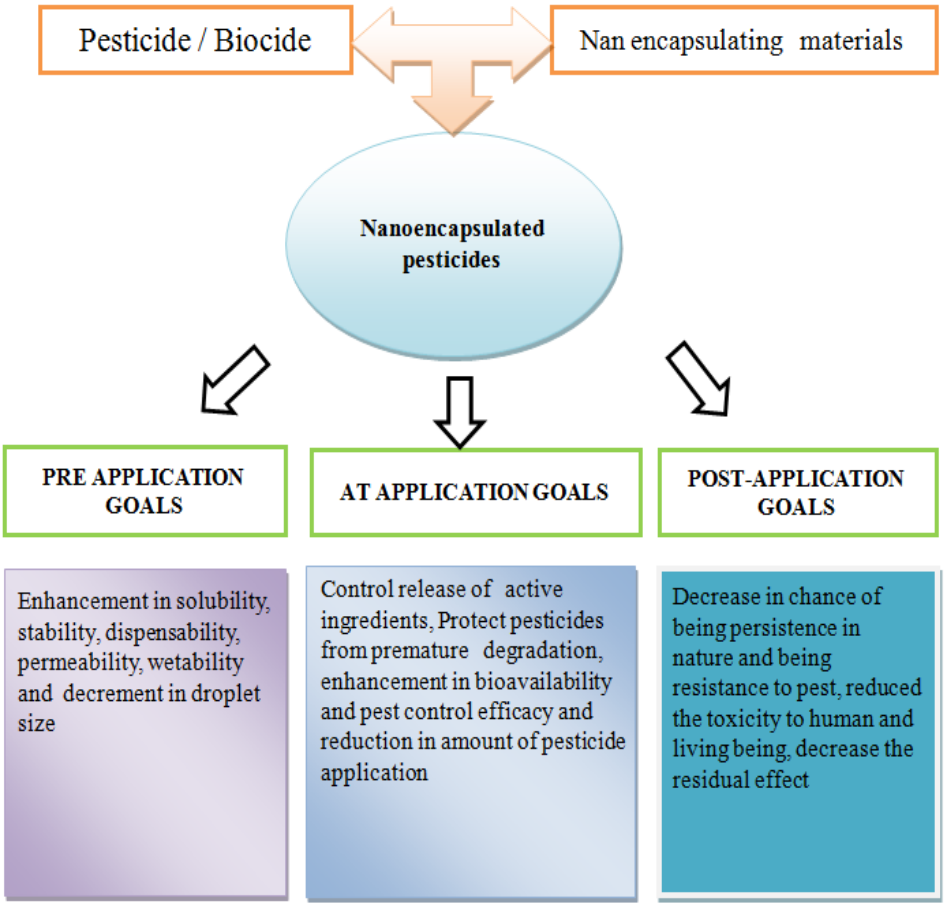


Figure 1.7 Key goals of nanotechnology in agriculture (Nuruzzaman et al., 2016)

Table 1.5: Principles studies reported to application of nanotechnology in agriculture

Year	Nanotechnology application	References
2006	Nano-TiO2 on glassy carbon electrode to detect parathion (pesticide) residue in vegetables	(Li et al., 2006)
2008	Starch nanoparticles conjugated with fluorescent material transporting DNA to transform plant cells	(Liu et al.,2008)
2009	PEG coated nanoparticles loaded with garlic essential oil for control of storage pests (*Tribolium castaneum*)	(Yang et al.,2009)
2011	Optical sensor for the detection of pesticides (Dipel, Siven 85% WP) in water using ZnCdSe Quantum dots films	(Baker et al.,2011)
2012	Neem oil (*Azadirachta indica*) nanoemulsion as larvicidal agent	(Anjali and Sharma, 2012)
2012	Macronutrient fertilizers coated with zinc oxide nanoparticles	(Milani et al., 2012)
2012	Amphotericin B nanodisks (AMB-NDs) for the treatment of fungal pathogens in chickpea and wheat plants	(Perez et al., 2012)
2014	Nanoformulation based on chitosan/tripolyphosphate nanoparticles loaded with paraquat herbicide for control release and eco-friendly weed management	(Grillo ., 2014)

1.19 Application of nanotechnology as pesticides delivery

The successful application of nanotechnology in biocontrol of pests achieved due to nanoparticles basic characteristics such as stiffness to plant leaves, permeability, thermal stability, crystallinity, reduction in repetitive pesticides doses (Bordes et al., 2009) and prevention in premature degradation (Nair et al., 2010). Table 1.4 represent some reports of successful role of nanotechnology in pesticides delivery. In nanoencapsulation, various natural and synthetic polymers such as chitosan, poly-ε-caprolactone, polyethylene glycol and sodium alginate as well as the block copolymers have served to encapsulate a wide

range of pesticides through the formation of different nano-range materials. Figure 1.8 represent various form of nanodevice prepared by different nanotechnological methods such as nanoprecipitation, ionic gelation, layer by layer, emulsion diffusion, solvent evaporation, double emulsification, emulsion coacervation (Boehm et al., 2003; Mora et al., 2010; Esmaeili and Saremnia, 2012)

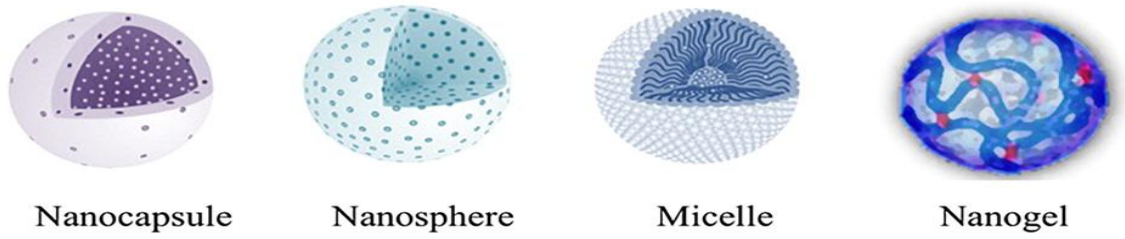


Figure 1.8: Major morphological form of nanoparticles employed to pesticides application

Table 1.6: Different nanoparticle forms used in delivery of pesticides or detection in pesticides residues

No.	**Nanomaterials**	**Morphological form**	**Application**	**References**
1.	Polymer based nanomaterial	Nanocapsules	Nanoencapsulation of imidacloprid resulted decreased in dosage and environmental pollution significantly	(Memarizadeh and Ghadamyari, 2014)
			Insecticidal activity against army worm retained over seven days	(Yin et al.,2012)
		Nanospheres	Aqueous suspension stable over two months of nanospheres	(Boehm et al., 2003)
		Micelles	Nanoemulsion formulation of β- cypermethrin exhibited better stability	(Wang et al., 2007)

			than commercial microemulsion of cypermethrin	
			Solubility of rotenone insecticides increased to 13000 times greater than that of free rotenone in water	(Lao et., 2010)
		Nanogel	Chitosan nanogels exhibited significant fumigant toxicity than the free oil control store grain pest over a longer period	(Bhagat et al., 2013)
2.	Lipid based nanomaterial	Liposomes	Pest control efficacy was observed for a longer period	(Hwang etal., 2011)
		Solid lipid Nps (SLPs)	Chitosan based SLPs demonstrated to protect deltamethrin against photodegradation	(Nguyen et al.,2012)
3	Porous inorganic nanoparticles	Mesoporous silica Nps	Sustain release of 2,4-D was performed till 26 days and up to 30 days for picloram	(Prado et al., 2011)
		Porous hollow silica nanoparticles	Control release of avermectin can be obtained by adjusting pH and temperature; UV-shielding properties were also improved	(Chen et al., 2004)
4.	Clay based nanomaterials	Clay materials	Ethofumesate insecticide slow-releasing properties were achieved due to clay/pesticideinteractions.	(Chen ., 2010)

1.20 Chitosan polymer in crop protection and production

Chitosan polymer has been used as sewage treatment till 1980; later it has explored in the field of agricultural with focusing wide applications such as plant growth regulator, seed coating agent, vegetable, fruit ant staling agent and soil conditioner. Schematic representation of Figure 1.9 indicate preparation and application of chitosan nanoparticles by different approaches. Several studies reported that chitosan has not only an antimicrobial activity but plant elicitor to acquire resistance against pathogens (No et al., 2007; Sharp, 2013).

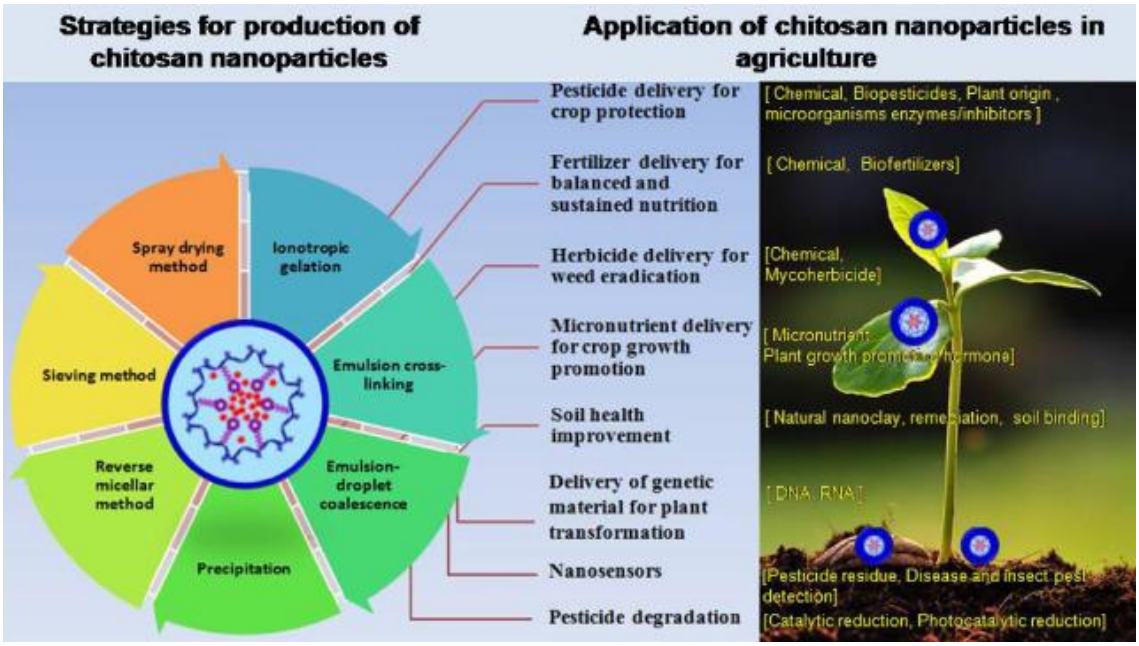


Figure 1.9: Various method for the production of chitosan nanoparticles and their applications as a delivery system in agriculture (Kashyap et al., 2015).

As well as, it has been reported that chitosan could act as plant growth and development promoting agent which ultimately help in enhancement of crop yield (Gornik and Gornik, 2008). It has also demonstrated that chitosan could play role in plant immune system in Tomato (Benhamou et al.,1992), cucumber (Ben-shalom et al., 2003), chilli seeds

(Photchanachai., 2006), strawberry (Ghaouth et al., 1992) and rose (Wojdyla et al., 2004). Moreover, chitosan expended as biocontrol agent particular to the control of plant pathogenic fungi Fusarium (Rabea et al.,2003) and gray molds (Aziz et al., 2006) in some crops as well as induced defense against rice blast pathogen *Magnoporthe grisea* (Rodriguez., 2007). Besides these, chitosan has other an important role as an inducer in seed germination of Cucumber, Chilli, pumpkin, and cabbage plant (Chandrkrachang et al., 2002). Also, chitosan has a significant effect on the growth of drought resistance rice plant (Boonlertnirun et al., 2007). From the above reported studies, it interpreted that chitosan can be used in a different way to enhance crop production and protection.

- **Aim and scope of present investigation**

Insecticidal Cry proteins of *Bacillus thuringiensis* have been used as spore-crystal formulation and transgenic plant since a couple of decades as a biocontrol of different pests feed on crops. However, development of pest resistance in the second generation of transgenic Bt plants or failure of Bt crops encouraged scientist to engineered novel Bt toxin. To address this, in vitro molecular evolution is technique of domain swapping between Cry toxins to minimize the development cross resistance of pests and N-terminal alpha helix-1 deletion in Cry toxins are successful approaches for enhancement of toxicity of Cry toxin against devastating pests of crops. Formulation of nanoencapsulation of the hybrid toxin prepared to checks its insecticidal activity against pest *H. armigera*.

1. Engineering of Cry toxins to enhance Cry toxin toxicity to devastating pest *H. armigera*

Construction of novel hybrid Bt toxin by exchange of coding regions of domain I or III among different *cry* genes have been performed previously by using different techniques such as in vitro method of recombination for the preparation of a library of chimeric genes. However, it requires the use of 5' phosphorylated oligonucleotides for amplification and use of λ exonuclease to generate DNA segment. Thus, it becomes cumbersome when handling some of *cry* genes. Another method is a generation of restriction sites by site-directed mutagenesis becomes time consuming, which is more random and needs screening of large number of recombinants. Thus, an appropriate method for exchange specific domain coding region among multiple cry genes is the need of present time. Overlap-extension PCR is a simple, versatile technique employed for the in vitro gene splicing. Currently, Nanotebiochnology is an emerging branch and has been

proved to successful application in agriculture. Thus, present research work with following objectives **:**

- Domain swapping of Cry9Aa toxin with Cry1Ac by overlap extension PCR to observe change in activity of toxins against *H. armigera*
- Investigation of N-terminal alpha helix-1 deletion in prepared Cry1Ac-Cry9Aa hybrid toxin to further improvement in toxicity to *H. armigera*
- Optimization of nanoencapsulation of Cry1Ac-Cry9Aa hybrid toxin in chitosan nanoparticles to retain insecticidal activity of toxin
- Preparation of silver nanoparticles and determined its insecticidal activity against *Spodoptera litoralis*

2. Preparation of Bt spore-crystal formulation to prevent UV radiation inactivation of spores and crystals

The spore and crystal of *Bacillus thuringiensis* are sensitive to UV radiation. Their activity declined during prolong exposure to UV radiation in the field. To retain their activities, formulation is a kind of process which can maintain the insecticidal activity of toxin in the field. Encapsulation is a type of formulation which has been proved to shown delivery and maintaining bioefficacy of active ingredients.

Objectives:

- Optimization of microencapsulation of *Btk HD-1* spore-crystal formulation by suspension cross-linking method
- Investigation of spore viability and crystal activity of formulated *Btk HD-1* spore-crystal upon UV radiation and field level efficacy of the formulation