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TOXICOLOGICAL STUDIES ON POPULATIONS OF COTTON LEAF WORM DURING SELECTION OF RECOMBINANTS IN *BACILLUS THURINGIENSIS* AS INSECTICIDAL AGENT

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ABSTRACT

The aim of this study was to induce recombinants in *Bacillus thuringiensis* species that are toxic to pests in the environment of Saudi Arabia . The lab performance of certain released *Bt* treated *Ricinus communis* for their effect on *Spodoptera litura* were evaluated in comparison with non *Bt* plant parts . Two Bacillus strains were used in this study belonging to *Bacillus thuringiensis* and *Bacillus subtilis* . These strains were genetically marked using antibiotics and crystal violet . The strains were conjugated depending on the opposite genetic markers . From the mating four recombinant transconjugants were isolated to be evaluated for insecticidal activity against the larvae of *Spodoptera litura* . Investigations of toxicological studies were therefore carried out to evaluate the potential role of *Bacillus thuringiensis* (Bt) recombinants as a natural pest control agent against *Spodoptera litura* . Two different formulations of the recombinant pathogens were tested for their efficacy against the pest . The results appeared that larvae feeding on Bt treated plants was weight decreased than non Bt treatment in control experiment . It can be concluded that *Bacillus thuringiensis* has a great potential for controlling and suppress the feeding of larvae on plant leaves . It is interesting to note that mortality, developmental rates and larval body weights were greater on the diet containing crystals + endospores than that in the diet treated with crystals . Average weight of larvae tolerated Bt intoxication appeared more weight increase than that in non Bt feeding . In addition , increased general average weight achieved that larval weight was increased in Bt sprayed plants with crystals + endospores than that in Bt sprayed with crystals alone . Accumulated larval mortality was increased in response to diet treated with crystals and endospores than that treated with crystals . This indicated that the diet treated with Bt containing crystals and endospores was more toxic than that containing crystals only . However , the ratio between consumption to the weight of surviving larvae was slower in all Bt treatment diet than that in non Bt treatment diet . while , the average of consumed diet treated with crystals + endospores was slower than that treated with crystals . On the other hand , the ratio between feed consumption to the weight of surviving larvae was greater in the diet treated with crystals + endospores than that treated with crystals . This indicated that larvae tolerated high toxic agents (crystals + endospores) was highly efficient in metabolism of diet to overcome diet toxic effects . These results indicated that there is a dire need to search and test more and more recombinant isolates of Bt for tracing out the best to achieve maximum control of cotton and vegetables leaf worm to reduce pesticides needed , because of historically, resistance to insecticides which has been a limiting factor for effective chemical control of cotton and vegetables pests in the world .

Keywords . Accumulative mortality, accumulative consumption , *Bacillus thuringiensis* , conjugation , recombinants , *Spodoptera littura* .

INTRODUCTION

Pest control in Kingdom of Saudi Arabia is entirely relied on the application of chemical agents . Little information is known about the natural presence of *Bacillus thuringiensis* species that possess insecticidal activity in the environment of Saudi Arabia Kingdom . It would be of interest to search for native species of toxic Bt strains that can be used in pest control management .*B. thuringiensis* was first discovered in 1901 by Japanese biologist Shigetane Ishiwatari. In 1911, *B. thuringiensis* was rediscovered in Germany by Ernst Berliner, who isolated it as the cause of a disease called *Schlaffsucht* in flour moth caterpillars. In 1976, Zakharyan reported the presence of a plasmid in a strain of *B. thuringiensis* and suggested the plasmid's involvement in endospore and crystal formation (Zakharyan *et. el.* [64 , 58]) . *B. thuringiensis* is closely related to *B.cereus*, a soil bacterium, and *B.anthracis*, the cause of anthrax: the three organisms differ mainly in their plasmids. Like other members of the genus, all three are aerobes capable of producing endospores [40] . Upon sporulation, *B. thuringiensis* forms crystals of proteinaceous insecticidal δ-endotoxins (called crystal proteins or Cry proteins) , which are encoded by *cry* genes [5] . In most strains of *B. thuringiensis* the *cry* genes are located on the plasmid [63] .

Cry toxins have specific activities against insect species of the orders Lepidoptera (moths and butterflies) , Diptera (flies and mosquitoes) , Coleoptera (beetles) , hymenoptera (wasps, bees, ants and sawflies) and nematodes. Thus, *B. thuringiensis* serves as an important reservoir of Cry toxins for production of biological insecticides and insect-resistant genetically modified crops. When insects ingest toxin crystals, the alkaline pH of their digestive tract activates the toxin. Cry toxin gets inserted into the insect gut cell membrane, forming a pore. The pore results in cell lysis and eventual death of the insect [6,15] . Gene transfer can generally be regarded as prokaryotic sex , or a mechanism to “mix genes” between prokaryotic community members . However, the process of prokaryotic gene transfer is dramatically different from sex as it occurs in dioecious higher organisms . Unlike sex in higher organisms, only one or a very limited number of closely linked loci are transferred at any one particular time. Entire genomes are never recombined, as for diploid organism. This leads to “micro-evolution of loci”. And certain loci are known to transfer more frequently than others.

Conjugation is a plasmid or transposon encoded mechanism of transfer that requires cell contact, first described by Lederburg and Tatum [25] . Because of their location on plasmids or transposons , the genes transferred in conjugation generally encode accessory functions such as antibiotic, UV, and heavy metal resistance or expanded metabolic capabilities (i.e. Xenobiotic degradation [62]). Conjugation has often been viewed as the most promiscuous of the three transfer mechanisms because of the lesser restriction on similarity of recipient to donor imposed by transformation and transduction .

Bacillus thuringiensis (hereafter abbreviated Bt) , a gram - positive sporulating bacillus, produces a proteinaceous parasporal crystal (delta-endotoxin) which is toxic against a number of lepidopterans and dipterans [10] . The gene(s) coding for crystal protein in different species and subspecies of BT are plasmid encoded [11] , although examples of chromosomal location of the gene(s) have also been reported [5] . The number of plasmids in different strains of BT varies from 2 to 12 with sizes ranging from *ca.* 1·5 to *ca.* 150 Md [11] . The crystal protein genes are mostly located in large plasmids (15–120 Md) [37] .

Toxin activity appears to be mediated by binding of toxins to cell membrane phospholipids [13] . Several studies revealed that the expression of the crystal protein gene is functionally related to sporulation specific events [34] and it was also found that one of the subunits of spore coat protein shares some similarities with the crystal protein [42] . However, desynchronization of sporulation and crystal formation could be achieved by short-term temperature shift-up or addition of chloramphenicol [42] or erythromycin [57] . This latter finding evokes questions about the correlation between sporulation and crystal protein gene expression .

Bacillus thuringiensis (Bt) has been , for more than 60 years, the most widely utilized biopesticide for the control of plant damages caused by insects [41] . These spore-forming gram-positive bacteria produce, during their stationary phase, a large crystal inclusion composed of proteins, the d-endotoxins or Cry proteins, which have a larvicide's activity on different insect species [26] . A variety of strains produce various d-endotoxins, each characterized by a narrow specificity, which serve as the basis of commercial preparations. This specificity, which allows the preservation of non-target insects and makes the preparations non-toxic for mammals, bird or fish, is one of the advantages presented by Bt preparations over chemical pesticides, another one being their lower capacity to induce resistance. However, because Bt preparations are very unstable under natural environment and present a narrow host range, multiple applications may be necessary during the crop growth period, which represents an inconvenience for users. To overcome these problems, approaches that have been taken include the construction of strains with enlarged host range or increased activity and, with the aim of delivering the Bt toxin in the vicinity of the targeted insect, the introduction of Bt toxin genes into plant associated bacteria or directly into the plant genome. Bt toxin genes have been introduced in a number of plant-associated bacteria such as root colonizers , *Azospirillum* , *Pseudomonas* spp. , symbiotic bacteria, *Rhizobium leguminosarum,* or endophytic bacteria , *Herbaspirillum seropedicae* and *Clavibacter xyli* subsp. *cynodontis* .With the exception of this latter species used in field releases with corn , the recombinant bacteria have been used mainly to produce Bt endotoxin preparations free of Bt spore [41] .

The multi-step toxicity process (Figure 1) includes ingestion of the Cry protein by a susceptible insect, solubilization, and processing from a protoxin to an activated toxin core in the insect digestive fluid. The toxin core travels across the peritrophic matrix and binds to specific receptors called cadherins on the brush border membrane of the gut cells. Toxin binding to cadherin proteins results in activation of an oncotic cell death pathway and / or formation of toxin oligomers that bind to GPI-anchored proteins and concentrate on regions of the cell membrane called lipid rafts . Accumulation of toxin oligomers results in toxin insertion in the membrane, pore formation, osmotic cell shock, and ultimately insect death. Whether oncosis , pore formation and / or both mechanisms are ultimately responsible for enterocyte death is still controversial .

Tansconjugant efficiency is the occurrence of a superior offspring from mixing the genetic contributions of its parents. These effects can be due to Mendelian or non-Mendelian inheritance. Two competing hypotheses, not necessarily mutually exclusive, have been developed to explain transconjugant efficiency. The dominance hypothesis attributes the superiority of hybrids to the suppression of undesirable (deleterious) recessive alleles from one parent by dominant

alleles from the other. It attributes the poor performance of inbred strains to the loss of genetic diversity, with the strains becoming purely homozygous deleterious alleles at many loci. The overdominance hypothesis states that some combinations of alleles (which can be obtained by crossing two inbred strains) are especially advantageous when paired in a heterozygous individual. The concept of heterozygote advantage / overdominance is not restricted to hybrid lineages . This hypothesis is commonly invoked to explain the persistence of many alleles (most famously the erythrocyte - sickling allele) that are harmful in homozygotes ; in normal circumstances, such harmful alleles would be removed from a population through the process of natural selection . Like the dominance hypothesis, it attributes the poor performance of many inbred strains to a high frequency of these harmful recessive alleles and the associated high frequency of homozygous - recessive genotypes [62].

The present study was undertaken to evaluate the expression of toxicity through combined several traits into a single bacterium to be expressed in recombinant isolates to increased insecticidal activity of Bt recombinants against cotton leaf worm. Though, accumulated consumption, accumulated mortality, larval weight , were evaluated as a results of larvicidial activity of recombinant proteins from *Bacillus* transconjugants .

MATERIALS AND METHODS

Microbial strains :

Bacillus thuringiensis serovar *Kurstaki* (NRRL HD-1) and *Bacillus subtilis* (NRRL NRS-744) were obtained from Dr. L.K. Nakamura, U.S. Department of Agriculture, Agricultural Research Service, U.S. Department of Agriculture, Peoria, I Ilinois. The strains were maintained on L.B. Slape medium, containing ; 5% peptone , 0.1% yeast extract and 0.5% NaCl , pH 7.5 [52] **.**

Media :

Bacillus thuringiensis strains were grown on T_3 medium (L^{-1} : 3 and 2 g tryptose , 1.5 g yeast extract , 0.05 M sodium phosphate , pH 6.8 and 0.005 g $MnCl_2$, according to Travers *et al* [60] , until sporulation was complete . All strains were grown on L agar (per liter 10 g tryptone , 5 g yeast extract , 5 g NaCl and 15 g agar , according to Travers et al , [60] at 30^{o}C until they sporulated .

Mass culturing of *S. litura*

The field collected egg masses of *S. litura* were used to initiate the mass culturing under laboratory conditions. The egg masses were kept in the egg cage. After emergence, first instar larvae were weighted and transferred to Bt and non Bt leaves in the bottles . The residual leaves were weighted , changed and the faecal pellets removed from the container every 24h . The grown up larvae were weighted daily.

Host plants

Fresh leaves of *Ricinus communis* were collected daily , squares and middle leaves were used for the experiments . Leaves were cleaned and three grams were weighted and placed in each container daily .

***Bacillus thuringiensis* formulations used in the experiment :**

Two *Bacillus thuringiensis* preparations were used ; crystals + endospores , crystals , in liquid formulations using 200 µl of the suspension . The bio – insecticide were applied on 250 ml bottles as well as mixed with 3 grams of leaves as diet for larvae .

Antibiotic susceptibility assays :

Antibiotic susceptibility was measured by plate diffusion method with cultures grown to logarithmic growth phase in nutrient broth of LB medium. Bacterial suspension (0.2 ml) was mixed with 10 ml of LB agar medium in petri dishes. Wells (8 mm diameter) were

punched in the agar , using a stainless steel borer, and were filled with 0.1 ml of the antibiotic concentration. The plates were incubated overnight at 37°C and the diameter of resulting zones of inhibition was measured, three replicates were used for each bacterial strain, and concentration of antibiotics used [8] . Different antibiotics were used with the concentration of 400 µg/ml [53].

***rfa* mutation :**

Strains having the deep rough (*rfa*) character should be tested for crystal violet sensitivity . For the test, nutrient agar plates are seeded with cultures of the strains to be tested and a sterile filter paper disc containing crystal violet is placed on the surface of each seeded plate by pipette 10 µl of a 1 mg/ml solution of crystal violet to the center of sterile filter paper discs (1/4 inch). Invert the plate and incubate at 37°C. After 12 h incubation, a clear zone of inhibition (approximately 14 mm) appears around the disc indicating the presence of the *rfa* mutation which permits large molecules such as crystal violet to enter and kill the bacteria. Wild-type strains or strains containing the *gal* deletion are not inhibited because the crystal violet cannot penetrate the cell.

Mating in liquid broth

Donor and recipient cells were grown in Luria Bertani broth – LB [53] at 30°C, with aeration to logarithmic growth phase (10^7cfu.ml^{-1}). Enumeration of donor and recipient strains was done by plating on LB agar with appropriate antibiotic. Equal amounts of donor and recipient cells (250 ml) were combined in 7 ml of prewarmed LB broth, and incubated at 30°C with shaking at 40 rpm [4] . After 2 hours, 100 ml of the mixture was inoculated onto LB agar plates containing the same concentration of antibiotic markers . Single colonies appeared on selective medium were picked up and grown on Luria Bertani slant agar [23] . Mating between *Bacillus* strains have been reported involving transference of small and large plasmids [8 , 32] . When grown in broth culture, *B. thuringiensis* strains are able to transfer plasmids in variable frequencies by a conjugation-like plasmid transfer process as first described by Gonzalez *et al*. [24] .

Separation of crystals and endospores :

Crystals and endospores were collected and purified according to Karamanlidou *et al*., [31] . Bacteria were grown in petri dishes and the spores were collected from nutrient agar washed three times in ice-cold distilled water. Pellets (spores and crystals) were resuspended in small volumes of distilled water . Bacterial cells were lysed to releasing spores and crystals and then collected by centrifugation (10000 x g for 10 min.) . Pellets were washed three times with ice-cold distilled waters and final pellets were resuspended in 20 ml of water and stored at -5°C. To purify crystals from spores and cellular debris, samples were sonicated and centrifuged on discontinuous sucrose density gradients (67 to 72 to 79% [wt/vol] sucrose) at 15000 xg for 2 h. Crystal bands and spore pellets were purified by three centrifugations and washed with distilled water. Final pellets were resuspended in small volumes of distilled water and stored at -5°C.

Bioassay of toxicity :

The toxicity was bioassayed with *Spodoptera littoralis* second instar larvae (mean body weight = 10 mg) according to Ignoffo *et al.* [28 , 29 , 30] with some modifications. Bacterial cell component of *B. thuringiensis* was approximately 10^9 crystals and/or spores per milliliter was used with the dilution of 1:1. larvae of *Spodoptera littoralis* were exposed to the appropriate dose of the component of *B. thuringiensis* using a Gentaur micropipette to dispense 200 µl of the suspension daily on three grams of diet surface of *Ricinus communis* to maintain an effective level than

control to be similar with transgenic plant toxins which are expressed continually, to overcoming some of the disadvantages of the conventional sprays [30] . Then this drop was evenly distributed over the diet surface with a sterile glass rod, and the surface was air-dried. Mortality was recorded daily after 24 h for six days. Surviving larvae from each replicate were pooled and weighted daily [30] .

Larval mortality of *S. litura*

Plant leaves of *Ricinus communis* were collected and sprayed with *Bacillus thuringiensis* preparations and non *Bt leaves* were used to feed the control larvae of *S. litura*. Plant parts of *Ricinus communis* were placed on moistened filter paper kept in 250 ml bottles and 10 second instar larvae were released in to each bottle . To avoid drying of plant parts, the filter paper was moistened at regular intervals. The leaves, bolls and squares were changed at 24 hr interval and the fecal pellets and dead larvae were removed from the bottles every 24 hr . The experiment was conducted under laboratory condition (28 ± 10C and 80 ± 5% RH) . Mortality was recorded at 24 hr intervals for six days . Bioassay technique was assayed against 4 days old larvae according to Dulmage [18] . Mortality percentage was calculated from the following formula ;

Mortality % = Number of mortality larvae in *Bt* treatment – Number of spontaneous mortality larvae in untreated control experiment / Initial number of surviving larvae in *Bt* treated X 100 .

Daily observations were made to count the surviving larvae . Actual values for larval mortality percent were estimated using the following equation [1] .

% Effect = (1- Nt / Nc) x 100

Where: Nt and Nc is the number of alive larvae in treatment (*Bt Ricinus communis*) and check (non Bt) , respectively.

In addition , the weight of surviving larvae (g) was calculated as follows :

Weight of surviving larvae (g) = Total weight of all of surviving larvae per bottle / Number of survivors per bottle .

Measuring accumulative increase in larval weight :

It was expressed as an increase in the weight performance of Bt leaves-feeding larvae over the weight of origin larvae . It was calculated using the following formula ;

Accumulative increase in larval weight = Average weight of treated larvae - Average weight of origin larvae / Average weight of origin larvae .

Results and Discussions

During sporulation many Bt strains produce crystal proteins (proteinaceous inclusions) , called δ-endotoxins, that have insecticidal action. This has led to their use as insecticides, and more recently genetically modified crops were used Bt genes. Spores and crystalline insecticidal proteins produced by *B. thuringiensis* have been used to control insect pests since the 1920 [36] . They are now used as specific insecticides under trade names such as Dipel and Thuricide. Because of their specificity, these pesticides are regarded as environmentally friendly, with little or no effect on humans, wildlife, pollinators, and most other beneficial insects .

Perhaps the major advantage is that Bt is essentially nontoxic to people, pets and wildlife. This high margin of safety recommends its use on food crops or in other sensitive sites where pesticide use can cause adverse effects. The insecticidal activity of Bt was first discovered in 1911. However, it was not commercially available until the 1950's. In recent years, there has been tremendous renewed interest in Bt and several new products are available.

The results in Table (1) appeared that accumulative consumption of surviving larvae

on all Bt treated leaves was less than that in non Bt treated leaves . This indicated that Bt preparations were reduced larval efficiency in feeding (induced some of starvation) when exposure to Bt formulations . General average of consumed leaves sprayed with crystal + endospores was less than that sprayed with crystals alone . These results revealed that crystals + endospores were more efficient in reduced the rate of leaves consumed by cotton leaf worm in relation to the leaves sprayed with crystals alone . It can be concluded that the bioinsecticides containing crystals + endospores from *Bacillus thuringiensis* and their recombinant isolates has a great potential effect for controlling and suppress the feeding of larvae on host plant leaves . There is a dire need to search and test more and more recombinant isolates for tracing out the best to achieve maximum control in cotton and vegetables leaf worm .This was represented by a lower feeding rate , higher mortality and a slower developmental of larvae maintained on transgenic or sprayed plants when compared to insects maintained on control plants . The laboratory evaluation of certain released *Bt* plants in comparison with their non *Bt* versions revealed that there was wide variation among the different plants on the rate of consumption and mortality , as well of *S. litura* .

These results agreed with Bagade *et al.* [7] , who reported that transgenic *Bt* cotton was found effective against three bollworms (*H. armigera, Earias spp.* and *P.gossypiella*) , as well as , Luttrell *et al.* [39] reported more tolerance in *S. frugiperda* against Cry 1 Ac than other bollworms . In addition , Burges [10] , found that *Bacillus thuringiensis* , a gram-positive sporulating bacillus, produces a proteinaceous parasporal crystal (delta - endotoxin) which is toxic against a number of lepidopterans and dipterans . The gene (s) coding for crystal protein in different species and subspecies of Bt are plasmid encoded [11] although examples of chromosomal location of the gene(s) have also been reported [5] . The number of plasmids in different strains of Bt varies from 2 to 12 with sizes ranging from *ca.* 1·5 to *ca.* 150 Md [11] . The crystal protein genes are mostly located in large plasmids (15 – 120 Md) [37] .

Previous screening failed to demonstrate highly active strains of *B. thuringiensis* against the cotton leaf worm [44] . In the present work, crystals + endospores from four recombinant isolates demonstrated a strong activity against feeding larvae of *S. littoralis* because it was reducing the rate of consumption by 36% than crystals .

Furthermore, larvae which have ingested sublethal doses of *B.t.* were slower in their development and leaf consumption, as also found by Ignoffo *et al.* [33] . The probability of such larvae to survive in field conditions is low and the surviving ones will be very slow in yielding subsequent generations [7].

As shown from the results presented in Table 2 that the larvae feeding on non Bt plants appeared a gradually weight increase . However the average weight of larvae tolerated Bt intoxication appeared more weight increase than that in non Bt feeding . In addition , the general average of increased weight achieved that larval weight in response to Bt sprayed plants with crystals + endospores was higher than that in Bt sprayed plants with crystals alone . This indicated that the larvae could tolerated more Bt toxicity composed of crystals + endospores , could appeared greater increase in their weights than the rate of weight affected by less toxic pesticide composed of crystals . Then the rate of weight increased in living larvae under more toxic Bt was greater than that of living larvae under the influence of less toxic Bt biopesticide . When reared on toxic diet containing crystals + endospores or crystals ,

the tolerated and resistant larvae grew and developed well was found on the diet containing crystals + endospores than that containing crystals , however , weight developmental rate of survivors was well at more toxic Bt preparation . The current study agreed with Huang et al. [27] , who suggested that the two Bt preparations are similar in fitness, apart from for resistance and that this resistance did not appear to carry a fitness cost . However, the similarity between the two Bt intoxication on diet may not completely to explain the fitness of the larvae at both treatments. The susceptible larvae could have reduced fitness. It is interesting to note that mortality, developmental rates and body weights of surviving larvae were greater on the diet containing crystals + endospores than that containing crystals .

As shown from the results presented in Table 3 that accumulated larval mortality was increased in response to diet treated with crystals and endospores than that treated with crystals . This indicated that diet treated with Bt containing crystals and endospores was more toxic than that containing crystals only.

Mortality obtained of the infected larvae may be due to the undigestion of the ingested food, or due to paralysis and/or the physiological disturbance due to the toxicity of the haemolymph [38] . *B. thuringiensis* strains produce at least two endotoxins that affect insect larvae. One of the toxins is associated with endospore coat, while the other is found in parasporal bodies (crystals) . The toxic material in parasporal bodies is a crystalline protein [12].

The present data revealed greater mortality of larvae after selection pressure to crystals and endospores more than that of crystals. These results agreed with that recorded by Sneh *et al.*, [56] , who found no development of true resistance in *S. littoralis* after 10 generations of selection by *Bt.* Entomocidus . On the other hand, unsuccessful attempts to select lepidopteran insects for *Bt* resistance has been reported by Moar *et al.* [43] , who found no significant increase in resistability in *S. littoralis* and S. *exigua* against *B. thuringiensis.* Information from resistance monitoring helps immensely in devising proactive resistance management strategies that can delay the rate of resistance development. The development of resistance to Bt toxin can be quite distinct, depending upon the species, selection pressure and geographical region. Hence, regular bioassays to assess the susceptibility of the test insect to Cry toxin will monitor the changes in a base line that can be used in monitoring resistance that may occur due to selection pressure of the Cry1Ac toxin.

Studies carried out by Gujar *et al.* [25] are also in accordance with the present findings who determined the baseline susceptibility of the American bollworm, *H. armigera* to *B. thuringiensis* var. kurstaki (B.t.k.) HD-1 and HD-73 strains for the populations collected from different places in India. The populations from Delhi, Raichur and Bangalore were least susceptible to the toxicity of B.t.k. HD-1. Whereas hyderabad and madurai populations were most susceptible. B.t.k. HD-1 caused neonate mortality of 37.4 per cent at 10 ppm and 68.6 per cent mortality at 100 ppm after 96 h of treatment. Mehna and Nagpur populations were least susceptible to B.t.k. HD-73 whereas Guntur, Bapatla, Hyderabad, Madurai and Vijayawada populations were most susceptible. HD-73 caused mortality of 62.3 per cent at 100 ppm and 91.7 per cent at 500 ppm after 96 h of treatment. Similarly, Kranthi *et al.* [33] reported that the baseline toxicity of CrylAc-endotoxin on field populations of the cotton bollworm, *H. armigera* through log dose probit analysis . LC50 values ranged from 0.07 to 0.99 µg/ml (14-fold) for CrylAa, 0.69 to 9.94 µg/ml (14-

fold) for CrylAb and 0.01 to 0.67 µg/ml of diet (67-fold) for CrylAc. Again, Gujar *et al.* [25] opined that there was no field level resistance in *H. armigera* against Cry1Ac protein and all the *H. armigera* populations were susceptible to Cry1Ac protein .

However, the increased mortality rates in laboratory feeding trials suggest a direct toxic effect of crystals and endospores . The results indicated that time of exposure enhanced the pest mortality. This agreed with Ahmedani et al [3] , who reported that none of Bt formulations exhibited 100% control of the best . Donovan *et al*., [16 , 17] found that a diet containing spores and δ-endotoxin of Bt isolates ; B- 21365, B-21366 and B-21367 was active against the larvae of other species of Coleoptera, including the red flour beetle, *T. castaneum* and the Japanese beetle, *Popillia japonica.* In addition , Nathan [48] , observed that infected larvae of *S. littoralis* with *Bt* induced a significant hyperproteinia .

The toxicity of *Bt* is due to the production of crystalline protein protoxins, known as δ-endotoxins [9] . Solubilized protoxins are activated by midgut proteases and bounded with the receptors of the epithelial cells [50] . The toxins insert themselves into the cell, where they form pores that lead to cell lysis, subsequently causing insect death [14] . Commercial *Bt* products generally consist of a mixture of spores and crystals, produced in large fermentors and applied as foliar sprays, much like synthetic insecticides. [54] .

Treatment of *S. Littoralis* larvae with crystals + endospores of the commercial formulations of *Bt* induced considerable changes in increasing mortality over that caused by crystals , increasing the weight of surviving larvae than that caused by crystals , this may be due to physiological , biochemical and developmental changes . Then , protein has been shown to affect important individual-level fitness-associated traits such as body size, growth rate, and fecundity; and at higher levels of organization has been linked to population dynamics, life histories, and even biological diversification [21] . Etebari [20] showed that many insecticides decreased feeding efficiency and protein amount of an insect's body .

Increase in accumulated mortality percentage of surviving larvae affected by crystals and endospores may attributed to stimulate synthesis of protein producing factors in insects as the protein requirement was increased for of *Bt* spores .

The present study also agreed with Muhammad Shoaib [46] , who found that mortality percentage of *T. castaneum* adults indicated statistically significant effect of Bt formulations used in the experiment , exposure time also exerted significant effect on mortality of the pest. There was a positive interaction between the exposure periods and Bt formulations. The findings of this study are in line with the work of Donovan *et al*., [16] , who found that *B. thuringiensis* strains contain novel crystal proteins which exhibit insecticidal activity against coleopteran insects including red flour beetle larvae (*T. castaneum*) and Japanese beetle larvae , *Popillia japonica* . Other scientists have also revealed significant effect of *B. thuringiensis* against *T. castaneum* [2] . An investigation into the histopathology of two isolates of *B. thuringiensis (*subsp. *indiana* and subsp. *Morrisoni*) , in the larval tissues of *T. castaneum* and *Plodia interpunctella* (Hubner) revealed that most changes caused by ingestion of the pathogens were localized in the midgut of the insects. Other effects were observed in muscles and tracheoles.

The results summarized in Tables 4 and 5 appeared that the ratio between consumption to the weight of surviving larvae was slower in all Bt treatment diet than that in non Bt treatment . This indicated the toxicity of Bt

formulations against cotton leaf worm leading to suppress feeding or inducing some of starvation in cotton leaf worm. However, the average of consumed diet treated with crystals + endospores was slower than that treated with crystals. This indicated that their was higher toxicity of crystals and endospores more than that of crystals , which is the current focus of this research . On the other hand , the ratio between feed consumption to the weight of surviving larvae was greater in the diet treated with crystals + endospores than that treated with crystals . This indicated that the larvae tolerated high toxic agents (crystals + endospores) was highly efficient in metabolism of diet to overcome more toxic effects in the diet . These results agreed with Salama *et al.* [52] ,who observed that the parasitoid *Microplitus demolitor Wilkinson* (Hymenoptera: Braconidae) , a parasitoid of the cotton leaf worm *S. littoralis* , was affected when feeding on hosts that had consumed a diet containing *B. thuringiensis.*

The results indicated that Bt insecticides consist of a mixture of spores and crystalline inclusion bodies that have to undergo a complex activation process to induce more lethal effects in susceptible insects than that caused by crystalline . Also, the conventional Bt insecticide degrades rapidly and requires sprays at regular intervals to maintain an effective level of control, whereas in the transgenic plant toxins are expressed continually. In transgenic plants, the Bt proteins are expressed continually as the activated toxin , thus overcoming some of the disadvantages of the conventional sprays. The rapid breakdown of the conventional microbial applications limits the temporal window of Bt exposure to herbivorous arthropods feeding on the target plant.

Peacock et al. [49] also found no significant differences in pupal and adult weight of several lepidopteran species sensitive to Bt toxin, although they observed higher mortality and longer developmental periods. Moreau and Bauce [45] reported a compensation of Bt effects with a longer developmental time and heavier pupa in *Choristoneura fumiferana* (Clemens) (Lepidoptera) . Developmental polymorphism lead to supernumerary instars to com pensate for adverse effects of the Bt toxin [45] .

These results agreed with Vojtech *et al.* [61] , who found in a laboratory study on effect *B*t toxins against *S. littoralis* , that *S. littoralis* larvae when reared for their whole lifespan on a mixture of leaves and stems from 2 – 4 week old *Bt* maize plants, the *S. littoralis* survival, developmental times and larvae weights were significantly affected by *Bt* maize. Also, Dutton *et al.* [19] , reported that the insects fed on either transgenic or *Bt* sprayed plants were negatively affected. Young *S. littoralis* larvae (1st and 2nd instars) were found to be the most sensitive to the *Bt* toxin [28, 29] .

The biocides *(B. thuringiernsis)* used in the present study caused considerable toxic effects against 2nd instar larvae of cotton leafworm . These results clearly indicated that , the different applied concentration of the bioinsecticides clearly affected the percentage of larval mortality , leaves consumption and larval weight , as well as , the ratio between consumption to the larval weight .

These results are in harmony with those obtained by Narayanan [47] . It may be possible in this instance to control flies by the use of this bacterium which incorporate spores and crystals of the appropriate strain of B.t.i. [22]. In addition, Qaim and Zilberman [51] reported that Bt cotton contains *CrylAc* gene which provides a fairly high degree of resistance to *H. armigera*, *Earias vittella*, and *P. gossypiella*, all of which are major insect pests in India. Although this assumption needs to be corroborated by open-field studies, it is likely that a mean lethal time greater is too

long to be very useful in preventing economic losses on most of corps. The ineffectiveness in achieving rapid reduction of pest populations is one of the main target of the reasons why farmers are often averse to use these control agents [59] . Finally, sprays of Bt toxins could be tested in order to increase and enhance the efficacy of microbial products for *S. littoralis* management.

Current conclusion and Future Developments:

In conclusion, the specific activity of Bt generally is considered highly beneficial. Unlike most insecticides, Bt insecticides do not have a broad spectrum of activity, so they do not kill beneficial insects. This includes the natural enemies of insects (predators and parasites) , as well as beneficial pollinators, such as honeybees. Therefore, Bt integrates well with other natural controls. For example, using Bt to control corn borers in field corn has been stimulated by its ability to often avoid later spider mite problems. Mite outbreaks commonly result following destruction of their natural enemies by less selective treatments.

In the earlier decades, farmers were majorly dependent on synthetic pesticides which on continuous application , created serious adverse effects such as interference of chemicals with the plant metabolism process, soil infertility, increased insect infestation due to evolution of resistance etc. Currently, special concerns go into the agricultural sector. With increase in the global population, there are large numbers of mouths to be fed thereby causing strain to the natural resources. With the introduction of technology, development of various molecular biology techniques has rendered good service in the field of biotechnology. The introduction of Bt technology have helped to eliminate many insect infestation thereby providing the plant with complete protection. This was only possible due to the different molecular biology techniques currently available. As seen above, different strategies were implemented to genetically engineer Bt recombinants to be use for insect resistance into plant crops. To date, Bt toxin (cry protein) have proved to be a potent candidate in controlling insect infestation. Though they are beneficial in their way, certain challenges were posed to the agricultural sector such as evolution of insects resistant to Bt toxin. Different integrated pest management strategies , as well as , induction of Bt recombinants have evolved which have been incorporated into plants so as to delay the resistance in insects to Bt intoxication . Adoption of Bt technology in the current agricultural practices can definitely help in improving the production of major food crops thereby assuring the future generation a better world, a better tomorrow. It could be concluded that crystals and endospores has more greater biochemical and physiological affects on the development of *Spodoptera littoralis* larvae than the other commercial formulations, crystals . The difference in activity might be due to the presence or absence of biologically active endospores + Cry toxins, their relative amounts and additive/synergistic effect of these toxins in the formulations, and batches of products [55] **.** Crystals and endospores is the best using Bt biopesticide due to its efficacy against cotton leaf worm and harmless to human health and environment.

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Figures and Tables :

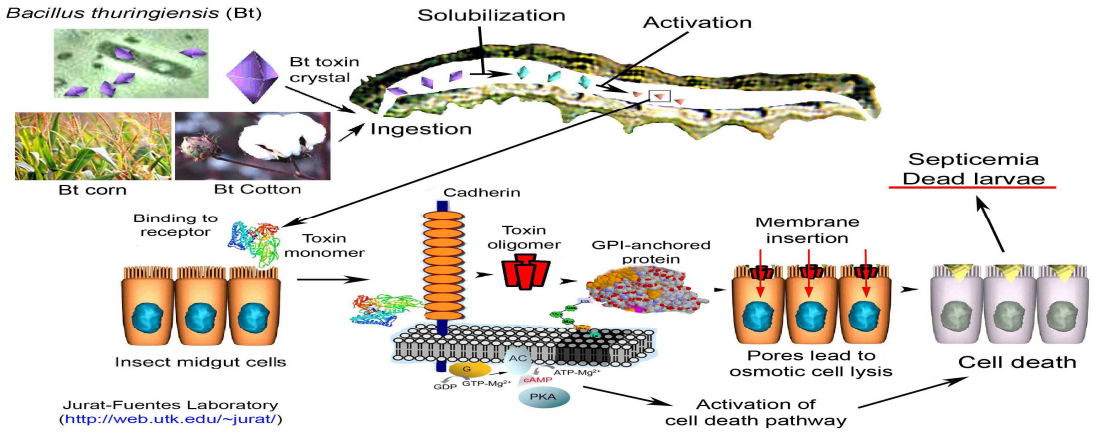

Figure 1 . Mode of action of Bt crystal protein in leaf worm .
Source : (http://web.utk.edu/~jurat/Btresearchtable.html) .

Table 1 . Accumulated consumption (gram / longevity larvae) of *Spodoptera littoralis* feeding on leaves of *Ricinus communis* sprayed with *Bacillus thuringiensis* preparations .

Time by hours	Bt preparations	Non Bt	*Bt*	*Bs*	T1	T2	T3	T4	Mean of Bt
24	Cry	0.18	0.18	0.19	0.18	0.19	0.19	0.17	0.18
	Cry + End		0.19	0.22	0.19	0.19	0.18	0.27	0.21
48	Cry	0.36	0.38	0.41	0.41	0.38	0.40	0.34	0.39
	Cry + End		0.38	0.45	0.39	0.48	0.45	0.56	0.45
72	Cry	0.54	0.59	0.63	0.63	0.56	0.54	0.55	0.58
	Cry + End		0.62	0.64	0.54	0.68	0.64	0.78	0.65
96	Cry	0.755	0.85	0.95	1.05	0.90	0.80	0.80	0.89
	Cry + End		0.90	0.87	0.81	0.94	0.85	1.16	0.92
120	Cry	0.895	1.17	1.29	1.38	1.38	1.24	1.14	1.27
	Cry + End		1.15	1.32	1.10	1.19	1.10	1.41	1.21
144	Cry	1.105	1.37	1.71	2.49	3.13	1.47	1.83	2.00
	Cry + End		1.35	1.72	1.51	1.27	1.31	1.62	1.46
Mean	Cry	0.639	0.23	0.29	0.42	0.52	0.24	0.31	0.335
	Cry + End		0.23	0.29	0.25	0.21	0.22	0.27	0.245

Table 2 . Increase in larval weight percentage of *Spodoptera littoralis* after feeding on leaves of *Ricinus communis* sprayed with *Bacillus thuringiensis* preparations .

Time by hours	Bt preparations	Non Bt	*Bt*	*B s*	T1	T2	T3	T4	Mean of Bt
24	Cry	66	123	78	131	170	121	77	116
	Cry + End		16	000	85	133	116	83	72
48	Cry	247	363	282	342	329	300	261	312
	Cry + End		650	400	457	616	550	533	434
72	Cry	871	810	773	757	805	742	811	783
	Cry + End		1650	2533	1800	2866	3016	3550	2569
96	Cry	1123	1436	1343	1568	1764	1278	1316	1450
	Cry + End		2700	2517	2185	1416	2500	3200	2419
120	Cry	1630	1888	1634	1842	2829	1984	2505	2113
	Cry + End		2616	4817	3185	1050	3350	3183	3033
144	Cry	1820	1419	1943	5410	10370	1584	4200	4154
	Cry + End		3000	3550	2728	166	3883	1950	2546
Mean	Cry	959	1006	1008	1554	2711	1001	1528	1468
	Cry + End		1772	2302	1740	1041	2235	2083	1862

Bt = *B. thuringiensis* *Bs = B. subtilis*

Table 3 . Accumulated larval mortality percentage of *Spodoptera littoralis* larvae after feeding on leaves of *Ricinus communis* sprayed with *Bacillus thuringiensis* preparations .

Time by hours	Bt preparations	*B. thuringiensis*	*B. subtilis*	T1	T2	T3	T4	Mean
24	Cry	00.00	04.17	0.00	4.17	8.33	0.00	2.78
	Cry + End	08.33	16.67	8.33	25.0	25.0	33.33	19.44
48	Cry	04.17	08.33	16.67	4.17	8.33	0.00	6.95
	Cry + End	25.00	25.00	20.83	41.67	37.5	41.67	31.95
72	Cry	04.17	12.50	20.83	4.17	9.08	9.08	9.97
	Cry + End	26.08	34.78	21.74	43.48	43.48	52.17	36.95
96	Cry	04.17	20.83	33.33	25.00	8.33	12.50	17.36
	Cry + End	42.85	38.09	47.63	57.14	57.14	41.67	47.42
120	Cry	12.50	37.50	37.50	66.67	16.67	50.00	36.81
	Cry + End	47.62	66.66	80.95	76.19	71.43	80.95	70.63
144	Cry	22.73	45.46	49.99	86.36	22.72	68.18	49.24
	Cry + End	84.21	73.68	100.0	100.0	78.94	84.17	86.83
Mean	Cry	7.96	21.47	26.39	31.76	12.24	23.29	20.52
	Cry + End	39.02	42.48	46.58	57.25	52.25	55.66	48.87

Table 4 . Ratio between accumulated consumption (g / longevity larvae) of *Ricinus communis* leaves sprayed with crystals isolated from *Bacillus thuringiensis* in relation to average weight of surviving larvae (g) of of *Spodoptera littoralis* .

Time by hours	Ratio	Non Bt	*Bt*	*Bs*	T1	T2	T3	T4	Mean of Bt
24	Consumption	0.180	0.18	0.19	0.18	0.19	0.19	0.17	0.18
	Weight	0.039	0.040	0.041	0.044	0.046	0.042	0.032	0.047
	Ratio	4.61	4.50	4.63	4.09	4.13	4.52	5.31	4.50
48	Consumption	0.360	0.38	0.41	0.41	0.38	0.40	0.34	0.39
	Weight	0.059	0.083	0.088	0.084	0.073	0.076	0.065	0.088
	Ratio	6.10	4.58	4.66	4.88	5.20	5.26	5.23	4.43
72	Consumption	0.540	0.59	0.63	0.63	0.56	0.54	0.55	0.58
	Weight	0.126	0.0163	0.201	0.163	0.154	0.160	0164	0.164
	Ratio	4.28	36.19	3.13	3.86	3.64	3.37	3.35	3.53
96	Consumption	0.755	0.85	0.95	1.05	0.90	0.80	0.80	0.89
	Weight	0.217	0.275	0.332	0.317	0.317	0.262	0.255	0.329
	Ratio	3.47	3.09	2.86	3.31	2.84	3.05	3.13	2.70
120	Consumption	0.895	1.17	1.29	1.38	1.38	1.24	1.14	1.27
	Weight	0.317	0.356	0.399	0.369	0.498	0.396	0.469	0.467
	Ratio	2.82	3.28	3.23	3.73	2.77	3.13	2.43	2.71
144	Consumption	1.105	1.37	1.71	2.49	3.13	1.47	1.83	2.00
	Weight	0.308	0.272	0.470	1.047	1.780	0.320	0.774	0.828
	Ratio	3.59	5.03	3.64	2.39	1.76	4.59	2.36	2.41
Mean	Consumption	0.639	0.23	0.29	0.42	0.52	0.24	0.31	0.335
	Weight	0.177	0.174	0.255	0.337	0.478	0.209	0.293	0.320
	Ratio	3.31	1.32	1.14	1.25	1.088	1.15	1.058	1.046

Consumption = Accumulated consumption

Table 5 . Ratio between accumulated consumption (g / longevity larvae) of *Ricinus communis* leaves sprayed with crystals + endospores isolated from *Bacillus thuringiensis* in relation to average weight of surviving larvae (g) of *Spodoptera littoralis* .

Time by hours	Bt preparations	Non Bt	*Bt*	*Bs*	T1	T2	T3	T4	Mean of Bt
24	Consumption	0.180	0.19	0.22	0.19	0.19	0.18	0.27	0.21
	Weight	0.011	0.007	0.006	0.013	0.014	0.013	0.011	0.013
	Ratio	16.36	27.14	36.66	14.61	13.57	13.84	24.54	16.15
48	Consumption	0.360	0.38	0.45	0.39	0.48	0.45	0.56	0.45
	Weight	0.032	0.045	0.030	0.039	0.043	0.039	0.038	0.044
	Ratio	11.25	8.44	15.0	10.0	11.16	11.54	14.74	10.23
72	Consumption	0.540	0.62	0.64	0.54	0.68	0.64	0.78	0.65
	Weight	0.105	0.105	0.158	0.133	0.178	0.187	0.219	0.181
	Ratio	5.14	5.90	4.05	4.06	3.82	3.42	3.56	3.59
96	Consumption	0.755	0.90	0.87	0.810	0.94	0.85	1.16	0.92
	Weight	0.109	0.168	0.157	0.160	0.091	0.156	0.198	0.173
	Ratio	6.93	5.36	5.54	5.06	10.33	5.45	5.85	5.32
120	Consumption	0.895	1.15	1.32	1.10	1.19	1.10	1.41	1.21
	Weight	0.150	0.163	0.295	0.230	0.069	0.207	0.197	0.217
	Ratio	5.97	7.055	4.47	4.78	17.25	5.31	7.16	5.57
144	Consumption	1.105	1.35	1.72	1.51	1.27	1.31	1.62	1.46
	Weight	0.184	0.186	0.219	0.198	0.016	0.239	0123	0.194
	Ratio	6.00	7.26	7.85	7.63	79.37	5.48	13.17	7.53
Mean	Consumption	0.639	0.23	0.29	0.25	0.21	0.22	0.27	0.245
	Weight	0.098	0.112	0.144	0.129	0.068	0.140	0.130	0.137
	Ratio	6.49	2.05	2.01	1.94	3.06	1.57	2.07	1.788

Consumption = Accumulated consumption