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The use of *Bacillus thuringiensis* toxins and spores for biological control of fall webworm *Hyphantria cunea* Drury (Lepidoptera:Erebidae)in Turkiye

Esraa Mahdı Al-Obaıdı [1] ، Prof. Dr. Ali İrfan İlbaş [2] ، Prof. Dr. Semih Yılmaz[2]

1 Department of Science, College of Mustansiriyah University, Baghdad, IRAQ
2 Department of Agricultural Sciences and Technologies, Erciyes University , Turkey

Abstract:

Biological control is the use of insect such as predators and parasitoids, or pathogens such as fungi and viruses, to control unwanted insects, weeds, or diseases. Biological control dates back to 324 BC, when Chinese growers were recorded using ants to feed on citrus pests. Our study aimed to determine the effects of local *Bacillus thuringiensis* (*Bt*) isolates and their Cry proteins on the larvae of *Hyphantria cunea*. Six strains of *Bt* were used: *Bt*SY49.1, *Bt*SY25.1, *Bt*SY33.3, *Bt*SY56.3, *Bt*SY27.1 and standard strain *Bacillus thuringiensis* var *kurstaki* (*Btk*). The larvae of *Hyphantria cunea* were collected from the Rize region of northeastern Turkey. Seven doses were determined for each strains at the level: 10 µg/ml: 50 µg/ml: 100 µg/ml: 250 µg/ml: 500 µg / ml, 1000 µg/ml: 2250 µg/ml. Different ages of larvae were exposed to *Bt* strains. Larval mortality was monitored for five days, included live and dead number of larvae. The result on the first day: SY25.1Bt isolates showed positive significant differences compared to other strains except for SY33.3Bt. on second day, SY25.1 Bt showed positive significant differences compared to SY56.5Bt and Btk except for SY27.1Bt, SY33.3Bt, SY49.1Bt. On the third day, SY27.1Bt showed positive significant differences compared to SY25.1Bt, SY33.3Bt, SY49.1Bt, SY56.5Bt and Btk. On the fourth day, SY27.1Bt showed positive significant differences compared to SY25.1Bt, SY33.3Bt, SY49.1Bt, SY56.5Bt and Btk. On fifth day, SY27.1Bt showed positive significant differences compared to SY25.1Bt, SY33.3Bt, SY49.1Bt, SY56.5Bt and Btk. We can have conclude the mortality rate of *Hyphantria cunea* increases with the experiment days in all used strains of Bt with all applied doses, and with the progression of time, the mortality rate increases with the higher dose used

Keywords: Biocontrol, *Hyphantriacunea*, *Bacillus thuringiensis*, *Bt*, Cry protein, Sustainable agriculture

Introduction:

Biological management using natural enemies benefits the ecology and reduces insect damage. Biological control uses parasites and predators to reduce populations to an economically damaging level (**Kergunteuil et al., 2016**). Natural insect pest foes include predators, competitors, parasites, and parasitoids. Their effectiveness in controlling long insect pests makes them biological control agents (**Bale et al., 2008**). Many biological control strategies require preserving natural enemies (**Settele et al., 2018**), (**Cloyd, 2020**). Pesticides are used to diminish natural enemy effects (**Dainese et al., 2017; Bommarco et al., 2011**). resources are supplied (**Holland et al., 2020; Landis et al., 2000**). new natural enemies are introduced (**Thomas and Reid, 2007**). or biological insecticides are discharged (**Evans, 2016**). Predators, parasitoid, pathogens, bacteria, fungi, viruses, oomycote, competitors, and parasitoids pathogens have been studied. Fall webworm moth *Hyphantria cunea* is most larva, an Erebidae moth, makes webbed nests on hard wood trees in autumn and late summer (**Ko, et al., 2015**).

Hyphantria cunea is found largely in Mexico,Canada, and elsewhere. *Hyphantria cunea* occupies Europe and most of Asia. *Hyphantria cunea* are live in massive webs they build on tree branches. These webs help organisms find mates, regulate temperature, and avoid predators, causing infection and predation (**Schmidt et al., 2003**).The world's most used biological pesticide *Bacillus thuringiensis* in addition to moth and butterfly guts, that found on leaves, ponds, animals,and flourmills (**Lacey et al., 2015**). Delta endotoxins, insect-killing crystal proteins, are produced by many Bt during sporulation. They have been utilized to make pesticides and, more recently, Bt-expressing crops to battle insect pests (**Daranas et al., 2019**). Cry proteins attach to articular receptors on midgut membranes of specific pests, rupturing them and killing insects. Some animals without gastrointestinal receptors cannot be affected by Cry protein and are not injured by Bt (**Thambugala et al., 2020**).

Bt produces two delta endotoxin crystals during sporulation. Insecticidal delta endotoxins are crystal proteins expressed by cry genes. Cytgeneses express proteins. Cry toxins are known to kill Lepidoptera, Diptera, Coleoptera, and Hymenoptera (**DiGiallonardo and Holmes 2015**). Bts R1, ashort RNA generated by Bt that binds to Cry5Ba RBS, may decrease toxin synthesis when the nematode is outside the host, enabling the worm to avoid behavioural defences. Silencing increases Caenorhabditis elegans' elegance microorganism consumption. After ingestion, BtsR1 expression decreases,

resulting in Cry5Ba toxin production and host death. Vegetative insecticidal proteins (Vip) were discovered in 1996 in Bt. Most VIP proteins do not compete for receptors since they do not have the same sequence. Compared to Cry proteins, certain Vip proteins skill insects (**de Andrade et al., 2020**). *Bt* were not insecticidal have para sporrans, a novel cry protein subtype discovered in 2000. Parasporal proteins with six subfamilies destroy cancer cells selectively without hemolysis (**Aguirre et al., 2021**).

An effort was made to incorporate the latest advances in Bt research into biological control of the agriculturally hazardous *Hyphantria cunea* and to determine which stages of the insect life cycle the biocide affects, from egg to adult and all ages. This was done to create *Hyphantria cunea*-controlling toxin for mutations.

Materıals And Methods:

Bacillus thuringiensis strains: *Bt* strains SY49.1 SY25.1, SY33.3, SY56.5, SY27.1, and *Bt kurstaki* were supplied as stock cultures and used in the current studies [111].

Activation of *Bt* strains and obtaining spore-crystal mixtures: *Bt* strains culture at -80°C was transferred liquid LB medium (pH 6.8±2) and incubated overnight at 37°C at 200 rpm. Then they were incubated at LB medium a second time to obtain fresh culture and used for sporulation studies. T3 liquids porulation medium (pH 6.8±2), containing tryptone (3g), tryptose (2g), yeast extract (1.5g), MnCl2, 0.005g, NaH2PO4, 6g, NaH2PO4, 7.1g preparedin 1liter distilled water were used after autoclaving for growing and obtaining more than 80% sporulation from the bacterial strains. Cultures were kept at 200 rpm and 37 °C for 4-7 day stoachieve at least 80% sporulation. The sporulated samples were centrifuged at 15,000 rpm at 4ºC for 10 minutes, and the spore-crystal mixture was precipitated and washed twice in 20 ml distilled water and centrifuged again.

The protein extraction method: Bacterial cultures were made in 3 L of Anderson medium at 30 °C in a 5 L bioreactor with a volumetric gas transfer coefficient (kla) of 13.32 h−1 Cells were grown for about 48 h or until approximately complete autolysis had occurred releasing the spores and the toxin crystals in the culture medium. The developed purification procedure was first used in an attempt to isolate the spherical crystals of H3. The pellet was then suspended in a 50 mL centrifuge tube with a saline solution, in order to enhance the hydrophobic interactions. An organicsolven (diethyl ether, di chloromethan eur hexane) was added to a ratio less or equal to 10%

(50, 75, or 100µL/mL of aqueous suspension) to minimize the risk of altering crystals. Finally the pellet was washed twice with cold distilled water [112].

The used Pest: The American white butterfly *Hyphantria cunea* (Drury) larvae were used to determinethe effectiveness of previously determined six local ***Bacillus thuringiensis*** strains toxins and their effect on thispestin different larval stages. Drury larvae were taken from Çaylee a town and district of Rize Province on the Black Sea coast of eastern Turkey, 18 km east of thecity of Rize. About 4000 caterpillars of different ages were collected from fields, orchids, hazelnuts and berries. And put them inside plastic boxes dedicated to collecting larvae, which are sterilized and prepared for storing larvae inside as shown in Figure (1).



Figure (1): Sample collection methods

Methods of keeping larvae in the laboratory: The larvae were kept in the laboratory in boxes designated at 24.9˚C and 68.5 % relative humidity, and after reaching the sixth (last) stage of life, the larva turned into a pupa, and after three months it began to turninto an adult. Feeding was done by artificial feeding consisting of 10 ml of natural honey mixed with 20 ml of distilled water to stimulate the female tolay eggs. Feeding was done by artificial feeding consisting of 10 ml of natural honey mixed with 20 ml of distilled water to stimulate the female to lay eggs. The female lays eggs for two days, after which the female dies immediately, and the eggs hatch within five days Figure (2).



Figure (2): *Hyphantria cunea* larvae in laboratories

Lethal effect of different isolates on *Hyphantria cunea* larvae: The last instar of the larva (sixth stage) was used to add six different isolates of Bt, and as follows Btk, SY49.1 Bt, SY25.1 Bt, SY33.3 Bt, SY56.5 Bt, and SY27.1 Bt, according to for the amount sindicated for each dose, seven doses were identified (coded as 1, 2, 3, 4, 5, 6, 7). Perstrain *Bt* and as follows: 10 µg/ml, 50 µg/ml, 100 µg/ml, 250 µg/ml, 500 µg/ml, 1000 µg/ml, 1/2 (2250) µg/mlon Subsequently, toxins (protein) were sprayed on plant leaves and placed inside dishes containing five larvae four repetitions for each dose. After that, the dishes were placed inside the incubator at a temperature of 25.3 ˚C and 53 % relative humidity. Lethal effects were determined and control have been compared. LD50 and LD99 using probit analysis after a five-day review values were calculated. During the period of conducting the experiment, work was carriedout inside the laboratories of the Faculty of Agriculture / Erciyes University and the data were recorded according to the

statistical analysis program spssversion 26 and the graphs according to the minitab program.

The lethal effect of a mixture of different isolates with spores on *Hyphantria cunea* larvae: The lastinstar of the larva (the sixth stage) was used to add three different isolates of Bt and as follows: Btk, SY49.1 Bt,SY27.1Bt, and seven doses of the mixture spores were applied and as follows: 10 µg/ml, 50 µg/ml, 100 µg/ml, 250 µg/ml, 500 µg/ml, 1000 µg/ml, 1/2 (2250) µg/ml on Subsequently. Mixture spores were sprayed on plant leaves and placed inside dishes containing five larvae four repetitions for each dose. Lethal effects were determined and control have been compared. LD50 and LD99 using probit analysis after a five-day review values were calculated.

The lethal effect of a mixture of different isolates *Bt* with spore crystal on *Hyphantria cunea* larvae: The third and fourth instar larvae (L3, L4) were used to add three different isolates of Bt as follows: Btk, SY49.1 Bt, SY27.1Bt and three different doses of the crystal spore mixture once and the crystal mixture again. It was applied as follows: 50 µg/ml, 250 µg/ml,1000µg/ml on Subsequently once and the crystal mixture was added to three doses again for each age stage mentioned previously. Spores of the mixture were sprayed on the plant leaves and placed in dishes containing ten larvae three repetitions for each dose. Lethal effectswere determined and control have been compared. LD50 and LD99 using probit analysis after a five-day review values were calculated.

Results:

The first day: SY25.1Bt showed positive significant differences compared to SY27.1Bt, SY33.3Bt, SY49.1Bt, SY56.5Bt and Btk, the differences were more than (LCD = 12.895) except for SY33.3Bt. While the other isolates did not show significant differences between them because their value was less than (LCD = 12.895), Where the lowest mortality rate in isolate was SY56.5Bt in the second, third, fourth, fifth and seventh doses, while the highest mortality rate was in isolate SY25.1Bt in the fourth and fifth doses, as shown in table (1) and Figure (3).

Table (1): the mortality rates for Cry proteins at different doses on H. Cunea larvae on the first day

Bt Isolates	Dose (µg/ml)							Mean BtIsolates
	10	50	100	250	500	1000	2250	
SY25.1Bt	10	5	15	40	40	25	30	23.57
SY27.1Bt	10	0	5	15	15	20	20	12.14
SY33.3Bt	10	30	5	20	5	25	0	13.57
SY49.1Bt	0	0	5	5	15	15	15	7.86
SY56.5Bt	15	0	0	0	0	5	0	2.86
Btk	5	0	5	0	0	5	10	3.57
MeanDose	8.33	5.83	5.83	13.33	12.5	15.83	12.5	
LSDIsolates		4.874	LSD Dose		5.264	LSD		12.895

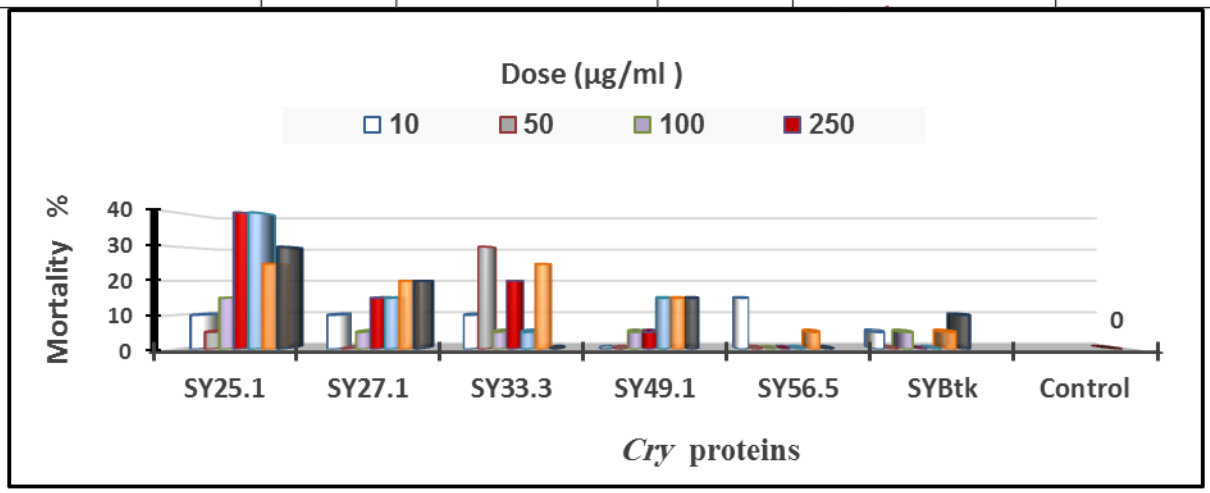


Figure (3): Relationship between the doses and mortality rates on H. Cunea larvae on the first day

The second day: SY25.1Bt showed positive significant differences compared to SY56.5Bt and Btk, the differences were more than (LCD = 18.15) except for SY27.1Bt, SY33.3Bt, SY49.1Bt While the other isolates did not show significant differences between them because their value was less than (LCD = 18.15). The lowest mortality rate was in the Btk in the second, fourth and fifth doses, while the highest mortality rate was in SY25.1Bt in the seventh dose, as shown in table (2) and Figure (4).

Table (2): the mortality rates for Cry proteins at different doses on *H. Cunea* larvae on the second day

*Bt*Isolates	Dose (µg/ml)							Mean*Bt*Isolates
	10	50	100	250	500	1000	2250	
SY25.1Bt	30	25	40	50	45	30	65	40.7
SY27.1Bt	25	30	30	25	25	25	55	30.7
SY33.3Bt	35	30	5	30	30	40	30	28.6
SY49.1Bt	0	25	40	30	35	35	25	27.1
SY56.5Bt	30	25	15	5	20	15	0	15.7
Btk	10	0	35	0	0	20	25	12.9
MeanDose	21.7	22.5	27.5	23.3	25.8	27.5	33.3	
LSD Isolates		6.86	LSD Dose		7.41	LSD Isolates*Dose		18.15

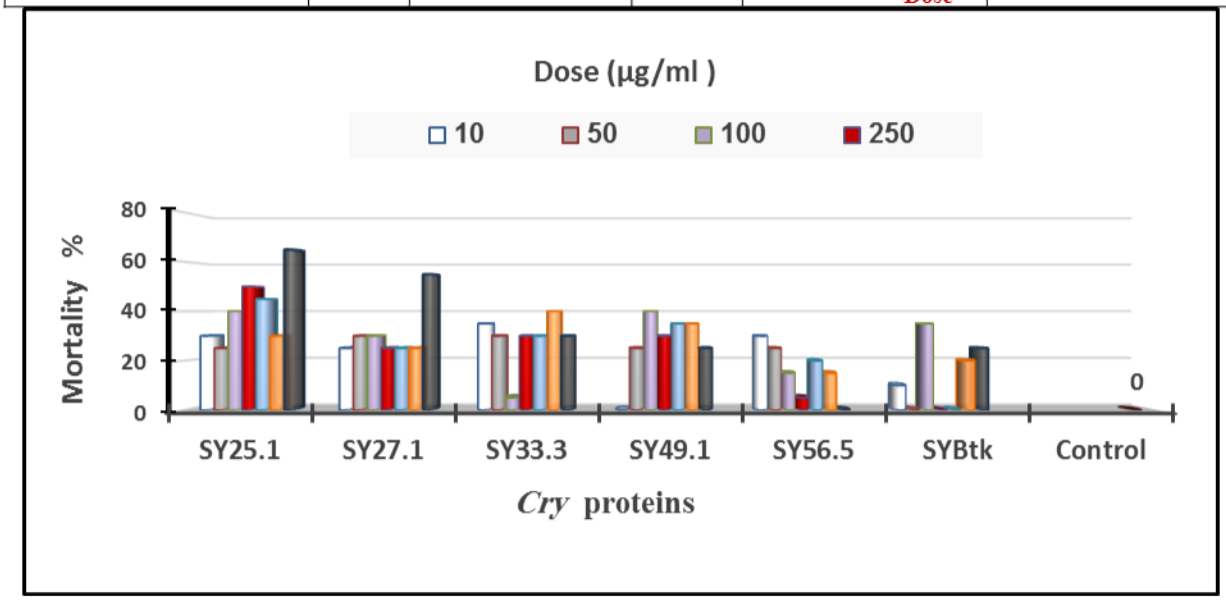


Figure (4): Relationship between the doses and mortality rates on *H. Cunea* larvae on the second day

The third day: SY27.1Bt showed positive significant differences compared toSY25.1Bt, SY33.3Bt, SY49.1Bt, SY56.5Bt and Btk, the differences were more than (LCD = 20.97). Where the lowest mortality rate was in Btk in the second dose, while the highest mortality rate was in SY27.1Bt in the fourth, and sixth doses, as shown in table (3) and Figure (5).

Table (3): the mortality rates for Cry proteins at different doses on *H. Cunea* larvae on the third day

*Bt*Isolates	Dose (µg/ml)							Mean *Bt*Isolates
	10	50	100	250	500	1000	2250	
SY25.1Bt	40	50	45	65	45	30	65	48.6
SY27.1Bt	50	60	50	90	80	90	80	71.4
SY33.3Bt	45	30	5	30	30	50	40	32.9
SY49.1Bt	30	70	80	50	80	95	85	70
SY56.5Bt	75	65	80	30	40	30	25	49.3
Btk	15	0	40	20	30	40	45	27.1
MeanDos	42.5	45.	50	47.5	50.8	55.8	56.7	
LSDIsolates		7.9	LSD Dose		8.56	LSDIsolates*Do		20.97

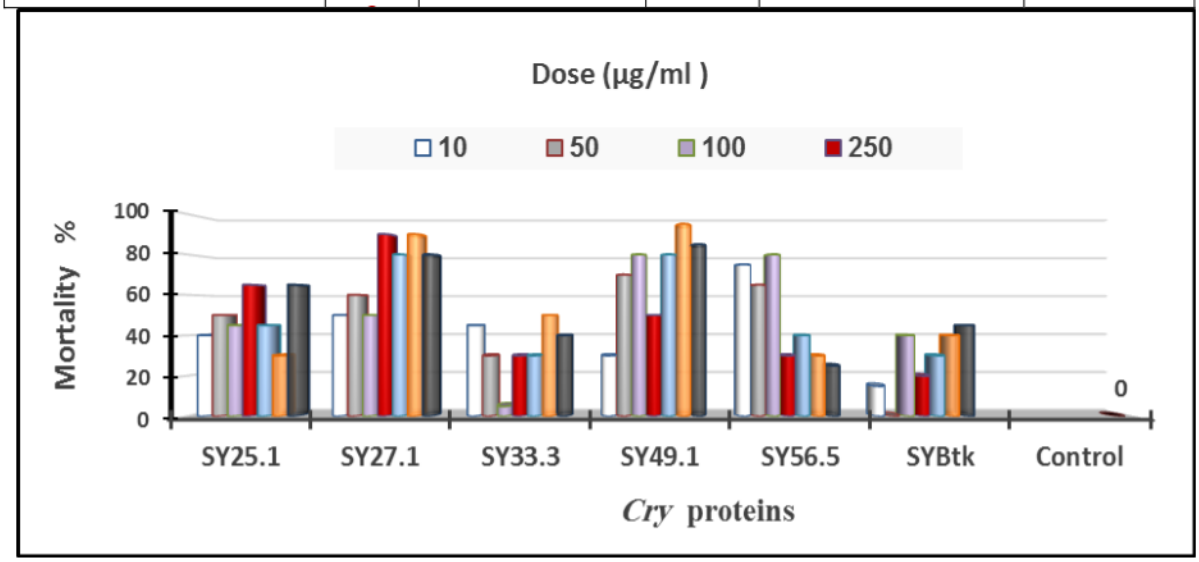


Figure (5): Relationship between the doses and mortality rates on *H. Cunea* larvae on the third day

The fourth day: SY27.1Bt showed positive significant differences compared to SY25.1Bt, SY33.3Bt, SY49.1Bt, SY56.5Bt and Btk, the differences were more than (LCD = 19.99). Where the lowest mortality rate was in SY33.3Bt in the third dose, while the highest mortality rate was in SY27.1Bt in the fourth, fifth and sixth doses, as shown in Table (4) and Figure (6).

Table (4): the mortality rates for the Cry proteins at different doses on *H. Cunea* larvae on the fourth day

*Bt*Isolates	Dose (µg/ml)							Mean *Bt*Isolates
	10	50	100	250	500	1000	2250	
SY25.1Bt	40	50	60	70	50	60	75	57.9
SY27.1Bt	70	90	85	100	100	100	95	91.4
SY33.3Bt	50	35	30	40	50	60	60	46.4
SY49.1Bt	35	95	95	95	90	95	95	85.7
SY56.5Bt	90	80	90	70	55	55	30	67.1
Btk	25	0	60	60	50	65	85	49.3
MeanDose	51.7	58.3	70	72.5	65.8	72.5	73.3	
LSDIsolates		7.55	LSD Dose		8.16	LSDIsolates*Dose		19.99

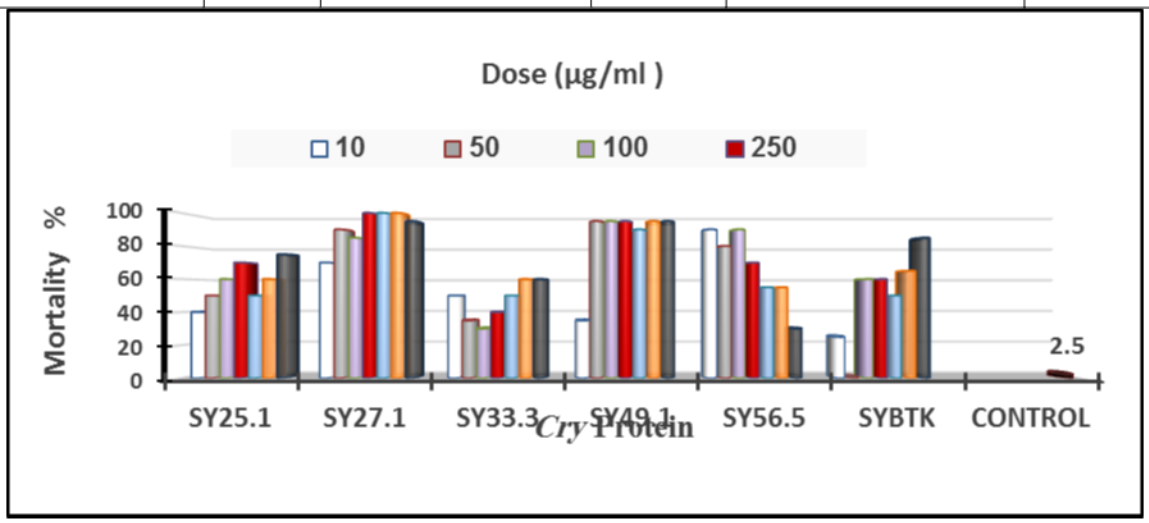


Figure (6): Relationship between the doses and mortality rates on *H. Cunea* larvae on the fourth day

The fifth day: SY27.1Bt showed positive significant differences compared to SY25.1Bt, SY33.3Bt, SY49.1Bt, SY56.5Bt and Btk, the differences were more than (LCD = 19.11). Where the lowest mortality rate was in Btk in the second dose, while the highest mortality rate was in SY27.1Bt in the third, fourth, fifth, sixth and seventh doses, as shown in Table (5) and Figure (7).

Table (5): the mortality rates for the Cry proteins at different doses on *H. Cunea* larvae on the fifth day

*Bt*Isolates	Dose (µg/ml)							Mean *Bt*Isolates
	10	50	100	250	500	1000	2250	
SY25.1	45	65	70	75	50	70	75	64.3
SY27.1	85	95	100	100	100	100	100	97.1
SY33.3	60	40	30	55	50	75	85	56.4
SY49.1	40	95	100	100	100	100	95	90
SY56.5	95	90	95	80	60	60	35	73.6
SYBtk	30	0	65	60	55	75	90	53.6
MeanDose	59.2	64.2	76.7	78.3	69.2	80	80	
LSD Isolates		7.22	LSD Dose		7.8	LSD		19.11

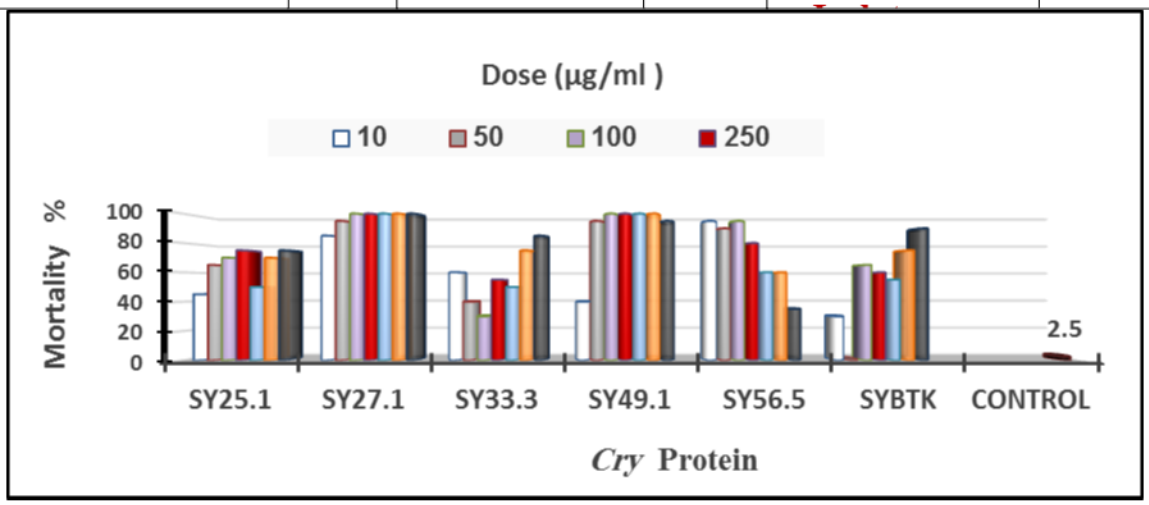


Figure (7): Relationship between the doses and mortality rates on *H. Cunea*larvae on the fifth day

Discussion:

The mortality Rate of *H. Cunea* by Bt is a particular kind of soild welling bacteria. It produces proteins that, when consumed by some insects, have a harmful effect on those insects, but not on others. Beneficial organism don't have receptors forcry proteins in their digestive tract and there fore the proteins remain inactive and do not pose any threat. *Bt* is not harmful to wildlife that is not its intended target (**Tomlin, 2009).** There are a variety of insect families that are susceptible to these sub species or strains. For examp lethe family of beetles, the family of flies including mosquitoes, and the family of butter flies are the insects that are targeted susceptible to some Bt toxins (**EllBendary, 2006).**

Hyphantria cunea is a moth of the family Erebidae. It is best known for the larval stage of its life cycle, which is responsible for building distinctive barenests in late summer and dropping to tree limbs. It is called a pest, but despite the fact that it does not cause any harmto trees, as the infected trees are generally in good condition and are not affected by the presence of thepest, and this has been attributed to of scientific studies in the field of agricultural pests and insects (**Tang et al., 2012).**

Based onour results, In all Bt strains and dosages, *Hyphantria cunea* mortality increases with days. Over time and at large doses, mortality rates rise. The extended lasting effect and toxicity of chemical pesticides have caused severe environmental problems. These include insecticide resistance in many vector species, mammalian toxicity, and pesticide aggregation in food. All these difficulties highlight the necessity for alternate bio-control agents. Effective yet risk-free biological control agents include Bt and *B. Sphaericus*. The extended lasting effect and toxicity of chemical pesticides have caused severe environmental problems. These include insecticide resistance in numerous vector species and pesticide aggregation in food. All these difficulties highlight the necessity for alternate bio-control agents. Effective yet risk-free biological control agents include Bt and *B. Sphaericus*.

They have garnered a lot of attention as potential alternatives to the chemical pesticides that are now on the market. Although microbial pesticides that are based on *Bt* and *B. Sphaericus* are available for use, large-scale use of the seproducts in under developed nations is impractical due to the high cost of the seproducts. The economic production of these two microorganisms by submerged fermentation and solid-state fermentation utilizing agro-industrial by-products and other wastes **(Argôlo-Filho and Loguercio, 2013).** There have been several efforts made by researchers to discover insecticides that are both safe and successful in the fight against economic pests. Pathogens have only been used to manage insect populations to a limited extent. Some of these agents have shown a great deal of promise and provide an out standing alter native to insecticides made from chemicals. *Bt* is an insecticide based on a bacterial pathogen, is now the most widely used kind of microbial pesticide (**Yılmaz, 2011).** *Bt* has shown to be an efficient bio pesticide due to the fact that it produces the proteins *Cry* and *Cyt*, both of which may be very lethal to insects in certain environments. *Bt* protects plants from the infection caused by pathogens. As a biological inhibitor that could stop plant disease, *Bt* was used in certain research studies (**Sanahuja et al., 2011).**

Despite the prevalence of many plant diseases, its agricultural impact on a wide range of crops, especially the dipteran order, is of paramount importance to mankind *Bt* is a pesticide bacterium that produces proteins that accrue in crystals with insecticidal and is widely used through out the biological control of insect pests such as Lepidoptera order. *Bt* is responsible for the formation of crystals with insecticidal effect (**Jung et al., 1995).**

When given orally, Btk detrimental effect on the digestive enzyme of *H. Cunea* and lowered the activity of enzymes of the digestive system in a dose-related manner, with the exception of beta-glycosidase and lipase. After treatment of the larvae, a rise in the pest's LDH level was observed (**Pinos et al., 2021).** Researchers in Korea were able to extract *Bt* from the grain dust of soybeans. Three proteins with different apparent molecular weights made up the d-endotoxin crystal that was produced by strain *Bt*-209. At 72 hours, *Bt*-209 demon strated a high level of lethality against *Hyphantria cunea* larvae, with a mortality rate of between 70% and 87% (**Tomlin, 2009).**

In *Hyphantria cunea* larvae, infected with Btk which exhibited the greatest gallotannin quantity, phenolic com pounds had an influence on the host defense and anti-oxidant enzyme activities. Inaddition, a decline was

seed in the levels of the flavonoid scatechin and rutin (**El- Bendary, 2006).** Insect mortality and bacterial growth in the gut and in the hemolymph were studied in *Hyphantria cunea*e to a commercial preparation of *Bt* and showed that *Hyphantria cunea* were killed rapidly by relatively low dosages (**Argôlo-Filho, and Loguercio, 2013).** Up on internal autopsy of the dead larvae, it was observed that *Hyphantria cunea* larvae died as a result of infection with *Bt*, and the number of viable cells of these bacteria increased 15-66 times (**Xu et al., 2017).**

*Bt is a spore-forming microorganism that is widely used as a bio control agent in agriculture. The insecticidal proteins that are secreted by Bt include toxins that are formed during the vegetative growth phase, parasporal crystalline –endotoxins that are produced during the vegetative stationary phase, and -exotoxins. There has been a vastvariety of Cry proteins discovered up to this point, and the majority of them have been classified as three-domain-Crytoxins, Bin-liketoxins, or Etx Mtx2-like toxins (**Osman et al., 1998).***

Bt efficient against leaf-chewing insects of Lepidoptera. The activity of pathogens against *Hyphantria cunea* of early instars was around 74 (7-100%). The strains of *Bt* were protective against leaf chewing insects (**Betz et al, 2000).**

Bt crystals have various forms (bipyramidal, cuboidal, flat rhomboid, or a composite with two or more crystal types). The crystal toxins are belonging to two structurally different groups (**Valtierra-de-Luis et al., 2020).** Bt is a bacterium that is found naturally in soils through out the world. To reproduce, Bt makes spores that grow into new vegetative cell. Bt spores have proteins that are toxic to insect larvae when eaten (**Jung et al., 1995).** *Bt* has been used as an effective bio insecticide because it produces the proteins Cry and Cyt, which are highly toxic to insects in certain situations. However, recently, *Bt* was used as a biological control agent that can suppress plant disease (**Xu et al., 2017).**

CONCLUSION: The mortality rate of *Hyphantria cunea* increased with the days in all the used *Bt* strains with all the used doses. The mortality rate increased; also, as the used dose is high, the mortality rate becomes more. *Bt* have potential effects on allstages of larvae, early and advanced stages of larvae of *Hyphantria cunea*; also, the extracted isolate and spore of *Bt* have lethal toxic effects on L3 and L4 larvae of *Hyphantria cunea.* Using Bt aginst

H. cunea in agricultural growing program will be effective and beneficial in terms of environment and human healts instead of using chamical preperate.

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