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Effect of *Bacillus Thuringiensis* and Sulfur on the Life of the Great Waxworm *Galleria mellonella*

Amjad Tayeh Ahimed[1,a], Merzah Hamzah Hadi[1,b], Adil Abaed Hassoni[1,c,*]
[1] Biological Control Department, Al-Mussaib Technical College, Al-Furat Al-Awsat Technical University, Iraq
[a] fgstqw231@gmail.com
[b] marzarussia@gmail.com
[c] dr.adil_aa@yahoo.com
[*]Corresponding author: dr.adil_aa@yahoo.com

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Abstract. *This study included the effect of Bacillus thuringiensis and sulfur on the life of the larval stages of the great waxworm. 100 soil samples were collected from different regions of Babylon Governorate to investigate the presence of Bacillus thuringiensis.According to the results shown by the study, it was found that the*the best method which used was more selective in isolating bacteria an inhibitor of bacterial spores by added sodium acetate, as the number of samples that contained *B. thuringiensis* bacteria was 57 samples from the total samples that were examined. *The results indicated that the amount of sulfur use had a significant effect on the destruction of the larvae of the great waxworm, as the concentration treatment (3 g) , reaching (85.0, 83.3% and 80.05%) after 48, 76 hours and a week, respectively***.** *The results of the current study indicated that the use of different concentrations of B. thuringiensis had a significant effect on the destruction of the larvae of the great waxworm, as the concentration treatment (10^{-2} g/ml^{-1}) significantly outperformed all the concentration treatments used in the research experiment. The highest rate (83.3, 84.95 and 85.0%) after 48, 76 hours and a week, respectively. The results showed that there were significant differences between its treatments caused by the two factors of the study (concentration - treatment method), as the interaction treatment (concentration 10^{-2}) with direct spraying achieved the highest rate for this trait of (90.0%) . The treatment of direct spraying outperformed the treatment of mixing with food, as it achieved the highest rate for the mentioned trait of (79.9%), while the treatment of mixing with food gave the lowest rate for this trait of (63.3%).*

Keywords: *Bacillus thuringiensis; sulphur; Galleria mellonella***.**

1. INTRODUCTION

The waxworm is the larva of the wax moth, and it belongs to the family of the Pyralidae. There are two types of it that humans keep commercially, they are the lesser wax moth *Achroia grisella* and the greater wax moth *Galleria mellonella*, The wax worm belongs to the Galleriini tribe under the snout moth family Galleriinae, and another species bearing the same name is the Indian meat moth, Plodia interpunctella, although this species is not commercially available. The great waxworm *G. mellonella* was first detected as an insect pest in Asia, but then spread to North Africa, Great Britain, some parts of Europe, North America, New Zealand, Latin America and Australia. It is expected that the pest will spread more all over the world, especially after the recent climatic changes that the world has witnessed [1], [2]. The infestation of the major wax worm causes great economic damage to the honeybee hives, the most important of which is the digging of silk tunnels in the wax discs, the presence of dense silk threads between the discs inside the hive in case of severe infection, noticing the excrement of wax worm larvae in the form of small dark pellets hanging from the silk threads inside the hive , Silk cocoons or their remains appear after the butterflies emerge from them, adhering to the woody parts inside the cell, the presence of damaged discs

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and waxy remains inside the cell, and watching the larvae themselves inside the tunnels in their different stages. Severe infestation of wax worms is called infestation as a result of wax worm infection, as this condition is seen in the brood discs when the bee's lap reaches the stage of the full insect and tries to get out of the hexagonal eyes, and it gnaws the wax covers, but it cannot leave the hexagonal eye as a result of falling into the trap of silk threads that spun it Wax worm larvae [3], [4].

Bacillus thuringiensis is a gram-positive, rod-shaped, aerobic, flagella-producing, soil-static, spore-producing bacteria commonly used as a biocide. *Bacillus thuringiensis* is commonly found in the intestines of the larvae of many species of moths and butterflies, as well as leaf surfaces, aquatic environments, insect droppings, insect-rich environments, mills and granaries [5], [6].

Sulfur powder is used as an insecticide, especially for spiders, to eliminate ticks and mites. Sulfur dioxide (SO2) is an atmospheric pollutant that is moderately persistent in the atmosphere and highly water soluble. SO2 may be transported, deposited, or transformed in various chemical reactions. SO2 participates in the sulfur biogeochemical cycle, which involves complex reactions of sulfur-containing compounds between abiotic and biotic components of ecosystems. Sulfur is one of the safe fungicides for human health, as it is fumigated on crops that are collected periodically, such as cucumbers, zucchini, tomatoes or beans. Therefore, the fungal control in previous periods depended largely on sulfur fumigation for its high effectiveness, and it is also considered an effective element to eliminate soil fungi such as Fusarium, Vertisilium and Rhizoctonia Therefore, adding it before plowing with the basic fertilizer and mixing it well is considered a strong fungicide from those diseases [7], [8].

2. MATERIALS AND METHODS

2.1 Culture media

For preparation of Culture media, all culture media were prepared according to the company's instructions.These culture media include: Nutrient Agar , MR-VP , T3 Agar , Sugers fermentation medium , Medium base .

2.2 Sample collection

100 soil samples were collected from Babylon Governorate represented the climatic variation and the difference in soil texture, with caution when taking samples from agricultural lands and making sure that *B. thuringiensis* preparations were not used in it by inquiring with the land owner . The surface layer of the soil was removed to a depth of 3-5 cm using a sterile knife (because the targeted bacteria are affected by ultraviolet rays, so they are not expected to be present in the surface layer) to a depth of 3 cm. The following on it (name of the area - nature of the land - date of taking the form) .

Then a code was given to each sample and kept in the refrigerator until the isolation process was carried out. Randomness was taken into account in sampling [9] and these procedures were applied with the collection of the collected samples.

2.3 Bacterial isolation and growth

The method for isolating *B. Thuringiensis* include Re-cultivation on nutrient media (NA) [10]. At 80% temperature for 15 minutes after adding sodium acetate [11].

2.4. Microscopic diagnosis

Examination of phenotypic traits **:** Based on what was mentioned [9].

2.5. Carbol Fuchsine

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The examination was conducted for colonies incubated for 72 - 150 hours and for positive colonies with Gram stain only to observe the presence of spores and crystals by preparing sterile and dry glass slides after marking the number of the colony to be examined. It was gently distributed and mixed with a drop of water and passed over a light flame several times to dry and left to dry at room temperature until it dried. Then, it was immersed in the basic fuchsin dye for 3-5 minutes, washed with distilled water, left to dry, and then examined with an oil immersion [9].

2.6. Biochemical test
The Biochemical test included: Methyl red test, Sugar Fermentation test, Voges – Proskauer test and Mannitol test [11].

2.7. Sulfur spray method
1- Weigh 2.5 gm raw sulfur in a sensitive balance .
2- It is added in a liter of distilled water for the purpose of dissolution with shaking to ensure the dissolution of sulfur.
3- Add the mixture to the spray container.
4- The process of spraying the beehive is carried out in the early morning or evening to avoid the heat of the sun's rays.
5- Observing and recording the results that appear as a result of the sulfur spraying process [12].

2.8. Preparation of the bacterial suspension
The method [13] was used. To prepare the bacterial suspension *B.thuringiensis*.

2.9. Great waxworm G.mellonella breeding
In order to obtain the first larval instars, the different roles of the insect were collected from tires infected with the major waxworm (L) *G. mellonella* from different apiaries. The adults and pupae were placed in wooden cages with dimensions 30 x 30 x 30 cm. Its base is made of wood, the sides of the wire mesh and the front of the muslin fabric sewn in a conical shape to insert the hand inside the cage, and on the ceiling of the cage a piece of cotton saturated with a sugar solution 100% was placed to feed the emerging adults and motivate them to lay eggs and for the purpose of obtaining the first larval instars, isolating the eggs placed on cardboard (subject). inside the cage for this purpose) From the raised adults, the cardboard was placed in sterile glass dishes, and after hatching, the first larval stage was transferred to sterilized petri dishes using a soft and moist mattress (with distilled water). Three replicates were used for each treatment, ten larvae for each replicate, 12 cm and 5 cm high dishes were used. Samples were sent. From adults of both sexes to the Natural History Museum, University of Baghdad for the purpose of confirming the diagnosis of the insect.

2.10. Statistical analysis
The statistical software [14] was used in analyzing the data, indicating the different treatments (isolates) in the studied traits, the larval death rate, and the significant differences between the means were compared with the LSD test.

3. RESULTS AND DISCUSSION

3.1 RESULTS

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3.1.1. Bacterial isolation

100 soil samples were collected from different areas in Babylon Governorate, and the presence of *B. thuringiensis* was investigated in the examined soil samples, as shown in table (1). As the isolates differed according to the geographical location of the area and the nature of the surrounding environmental conditions and the type of soil through the method that were used to isolate bacteria from soil samples. According to the results shown by the study, it was found that the method which used an inhibitor of bacterial spores which is sodium acetate was selective in isolating bacteria, as the number of samples that contained *B. thuringiensis* bacteria ,this method was 57 samples out of the total samples that were examined from the total samples.

Table 1. The percentages of bacteria presence in the soils of the regions of Babylon Governorate, according to the method of isolation

Site	first method			Second method		
	Number of models checked	number of samples that contained B.thuringiensis	%	Number of models checked	number of samples that contained B.thuringiensis	%
Nile	10	3	30	10	5	50
Albualwan	10	2	20	10	5	50
Albujaj	10	4	40	10	6	60
Mahaweel	10	4	40	10	8	80
Himyari	10	5	50	10	7	70
Hilla Center	10	4	30	10	4	40
Al Okir	10	4	30	10	6	60
Alwerdia	10	4	50	10	8	80
Alabara	10	3	20	10	5	50
Abu Ajarb	10	2	30	10	3	30
Total	100	35	% 35	100	57	% 57

The results of the current study, which are shown in table (1), proved that the method which used an inhibitor of bacterial spores, which is sodium acetate, for isolating bacteria is the best in obtaining the largest number of *B. thuringiensis* isolates, as it reached 57 isolates from 100 soil samples collected from different areas of Babylon Governorate.

3.1.2. Cultivational characteristics of B. thuringiensis.

The cultivar characteristics of the bacteria growing on the culture media for their growth were revealed, which consisted of Nutrient agar, Ammonium salt sugars and T3 Agar. It was found after the end of the incubation period that the colonies that were seen with the naked eye are white to yellowish-orange, small, round and mucous colonies, as several culture dishes were selected and their results were compared. As shown in table (2)

ISSN: 2789-6773

Table 2. Microscopic shapes of primary isolate colonies

colony symbol	microscopic description
A	white - diffuse - wavy and mucous
B	Bright yellow - small - round – smooth
C	white - diffuse - radial and rough
D	Bright orange - small - round and smooth
E	White - small - round and mucous

3.1.3. Phenotypic characterization of the crystalline protein of B. thuringiensis.

The results of the current study showed through microscopic examination of 57 bacterial isolates to investigate the shapes of crystalline protein using a 100× oil lens. 5 shapes of crystalline protein for these bacteria were distinguished: spherical, spherical and cubic , cubic, bipyramidal and oval as shown in table (3). Determining the percentage of crystalline forms present in the bacterial isolates. The results showed that the spherical shape was the most common among the isolates, as it recorded the highest percentage of (29.8%), while the lowest percentage was for the bipyramidal crystalline protein with a percentage of (12.2%).

Table 3. The percentage of crystalline forms of *B. thuringiensis* present in bacterial isolates

Crystalline protein shape	Total number of isolates	%
Spherical	17	29.8
Spherical and cube	13	22.8
Cubic	10	17.5
Oval	10	17.5
Bipyramidal	7	12.2
Total	57	

3.1.4. Effect of Sulfur on the life of the larval stages of the great waxworm

The results of the current study concluded that sulfur had a clear effect on the biological activity of the larval stages of the great waxworm. The results show in table (4) the clear effect of different concentrations of sulfur on the destruction of the larvae of the great waxworm after 48 hours.

Table 4. Effect of using sulfur on the mortality of the larvae of the Great Waxworm after 48 hours

Concentration g/L	direct spray %	mix with food%	Average %
Control	0.0	0.0	0.0
3	90.0	80.0	85.0
2	80.0	70.0	75.0
1	66.6	60.0	63.3
Average %	78.8	70.0	----
LSD values Concentration 6.94* , spray and mix 5.04* , Interference15.78*			
P(≤0.05)*			

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Table (5) shows the effect of sulfur on the numbers of larval deaths after 76 hours.

Table 5. The effect of using sulfur on the mortality of the larvae of the Great Waxworm after 76 hours

Concentration g/L	direct spray %	mix with food%	Average %
Control	0.0	0.0	0.0
3	86.6	80.0	83.3
2	70.0	70.0	70
1	63.3	60.0	61.6
Average %	73.3	70	----
LSD values Concentration 8.47* , spray and mix 6.33NS * , Interference14.09*			
P (≤0.05)			

It is noted from the results in table (6) that there is an effect on the preparation of the largest waxworm larvae after a week.

Table 6. Effect of using sulfur in the preparation of mortality of the larvae of the great waxworm after one week

Concentration g/L	direct spray %	mix with food%	Average %
Control	0.0	0.0	0.0
3	80.0	73.3	76.6
2	66.6	60.0	63.3
1	63.3	56.6	59.9
Average %	69.9	63.3	----
LSD values Concentration 8.92* , spray and mix 6.79* , Interference13.64*			
P (≤0.05) * ,			

3.1.5. The effect of the bacterial suspension of B.thuringiensis on the life of the larval stages of the Great Waxworm

When using different concentrations of the bacterial suspension of *B. thuringiensis* and testing its effectiveness on the larval stages of the great waxworm .

Table 7. Effect of different concentrations of *B. thuringiensis* in the destruction of the great waxworm larvae after 48 hours

Concentration mg/L $^{-1}$	direct spray %	mix with food%	Average %
Control	0.0	0.0	0.0
10^{-2}	96.6	70.0	83.3
10^{-5}	76.6	63.3	69.9
10^{-6}	66.6	56.6	61.6
Average %	79.9	63.3	----
LSD values Concentration 8.35* , spray and mix 6.20* , Interference14.07*			
P ≤0.05			

The results in table (7) indicate the obvious effect when using different concentrations of the bacterial suspension on the larvae of the great waxworm.

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Table 8. Effect of different concentrations of *B. thuringiensis* in the destruction of the great waxworm larvae after 76 hours

Concentration mg/L $^{-1}$	direct spray %	mix with food%	Average %
Control	0.0	0.0	0.0
10^{-2}	86.6	83.3	84.95
10^{-5}	73.3	70.0	71.65
10^{-6}	60.0	65.5	62.75
Average %	73.3	72.9	----
LSD values Concentration 7.25*,spray and mix 5.66* NS , Interference11.85*			
P ,(≤0.05)* NS : Non singficant			

As for the table (8), the results of the concentrations of the bacterial suspension that were used showed a clear effect on the destruction of the waxworm larvae after a week.

Table 9. The effect of different concentrations of *B. thuringiensis* in the destruction of the the great waxworm larvae after one week

Concentration mg/$^{L-1}$	direct spray %	mix with food%	Average %
Control	0.0	0.0	0.0
10^{-2}	90.0	80.0	85.0
10^{-5}	83.3	70.0	76.6
10^{-6}	76.6	66.6	71.6
Average %	83.3	72.2	----
LSD values Concentration 8.52*,spray and mix 6.08* , Interference13.69 *			
P ,(≤0.05)*			

During spore formation, many *B. thuringiensis* strains produce crystalline proteins (inclusions) called delta endotoxins (-endotoxins) that have an insecticidal action.
The results of the effect of different concentrations of *B. thuringiensis* suspension on the larvae of the largest waxworm showed that this bacteria had a clear effect on the destruction of the larval stages of the worm, according to the concentrations used in the experiment. By reviewing the studies and research in this field.

3.2. DISCUSSION

The results of the current study, which are shown in table (1), proved that the second method for isolating bacteria is the best in obtaining the largest number of *B. thuringiensis* isolates, as it reached 57 isolates from 100 soil samples collected from different areas of Babylon Governorate. These results are in agreement with a study of [15] who found that the second method using sodium acetate inhibitor at a concentration of 0.25 mol is the best method, as it excludes human pathogenic species, the traditional isolation method . From a total of 46,373 bacterial colonies of the genus Bacillus isolated from American soils, they found that only 250 isolates belong to *B.thuringiensis*. A study conducted by [16] in Spain showed that out of the total of 11982 colonies examined, only 1401 colonies belonged to *B. thuringiensis* **.**
By reviewing the sources and research of previous studies, it was found that the results of the current study agree with a study conducted by the researcher [17] in Turkey and the researcher [18] in Jordan. They found that the spherical shape of crystals is the most prevalent. While the current results did not agree with what was found by [19] in Iraq and Keshavarzi, that the bi-pyramidal form is the most prevalent.
The current study agrees with previous studies, as studies indicated that raw or microbial agricultural sulfur has a repellent effect on insects, as it acts as a repelling element for rodents (various types of worms

or suckers [20]. Many studies that have been conducted have shown that sulfur is a powerful fungicide for powdery mildew diseases and early or late blight if it is used by dusting as a treatment for these diseases, and it is dusted by using dusting or not. It is fumigated after spraying with one of the systemic fungicides [21].

The results of the effect of different concentrations of *B. thuringiensis* suspension on the larvae of the largest waxworm showed that this bacteria had a clear effect on the destruction of the larval stages of the worm . it was found that the results of our current study are in agreement with many previous studies. In a study conducted by [22] confirming a bioassay of *B. thuringiensis* on the Great Waxworm, a decrease in the appetite and food consumption of the larvae was observed as a result of infection with the bacteria, in addition to the appearance of black spots or areas on the body of the infected larvae, and these symptoms are similar to those of larvae. Wings when infected with the bacteria *B. thuringiensis*. After its death, its color changes from light to dark brown and then black. The larvae began to die 24 hours after the start of feeding, and the killing rate ranged after 72 hours (67.6 and 67.36%) in some bacterial isolates, and the death of larvae decreased in all isolates after 20 hours, while the effect of some isolates was 96 hours.

4. CONCLUSIONS

1-Presence of *Bacillus thuringiensis* in soil samples examined from different regions in Babylon Governorate. As the bacterial isolates differed according to the geographical location of the area, the nature of the surrounding environmental conditions and the type of soil.

2-sulfur had a clear effect on the biological activity of the larval stages of the great waxworm.

3- The use of different concentrations of *B. thuringiensis* suspension had a significant effect on the destruction of the larvae of the great waxworm.

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