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BIOLOGICAL ACTIVITY OF *BACILLUS THURINGIENSIS* AND GARLIC PLANT EXTRACTS ON THE WAXWORM *GALLERIA MELLONELLA* IN BABYLON PROVINCE

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Abstract

The current study was conducted of 100 soil samples from different areas in Babil Governorate, investigation analysis for bacteria *Bacillus thuringiensis***.** 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 more selective in isolating bacteria, as the number of samples that contained *B. thuringiensis* bacteria when using this method was 57 samples from the total samples that were examined. The concentration of water and alcoholic extracts of garlic plant had a significant effect on the destruction of the larvae of the Great Waxworm, as the concentration treatment (3000 mg/L^{-1}) significantly outperformed all the concentration treatments used in the research experiment and recorded the highest rate of (94.1, 94.9 and 79.9%) after 48, 76 hours and a week passed, 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%).

Key word: *Bacillus thuringiensis*, garlic plant*, Galleria mellonella*.

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 (Atlas, 2017; Kwadha *et al*., 2017). 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 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 (Desbois and Coote ,2012; Jorjao *et al* ., 2018) .

Bacillus thuringiensis (Bt) 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. *Cadra calidella* was also found to be a parasite on the dried fruit moth *Cadra calidella* in laboratory experiments. Many of the butterflies tested were infected with this parasite (Roh *et al.,* 2007; Kumar *et al.,* 2019) .

Allium sativum is a herbaceous monocotyledon that belongs to the genus Garlic. The sulfur compounds extracted from garlic can reduce the risk of chronic diseases, and the sulfur compounds found in whole garlic cloves are divided into two main types, namely, the amino acid allyl cysteine sulfoxides (γ-glutamyl cysteine). Garlic contains several non-sulfur chemical compounds, such as phytochemicals, the most important of which are flavonoids, steroid saponins, organic selenium compounds, and allixin), which work in cooperation with sulfur compounds (Irfan *et a*l. ., 2012; Rahat *et al.,* 2013).

Materials and methods

Culture media

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 .

Dyes and solutions

gram stain, fuchsin basic dye , Sodium ascent solution 0.5 mol .

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 (theiry and Frachon, 1997) and these procedures were applied with the collection of the collected samples.

bacterial isolation and growth

- first method It adopted a method (Ohba and Aizawa , 2009).
- Second method for isolating *B. thuringiensis*

Re-cultivation on nutrient media (NA). At 80% temperature for 15 minutes after adding sodium acetate (Travers *et al*., 1987).

Microscopic diagnosis

Examination of phenotypic traits : Based on what was mentioned (Thiery and Frachon, 1997).

Carbol Fuchsine

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 lens.

Biochemical test

The Biochemical test included : Motility test , Methyl red test , Sugar Fermentation test , Voges – Proskauer test , Mannitol and catalase test . .

Garlic extract

After grinding the garlic cloves, garlic extract was prepared by taking 300 gm of powder and placing it in a glass beaker with a capacity of 1000 ml, and then adding sterile distilled water to it. The plant material was mixed with an electric shaker for 15 minutes. Then the solution was left for one day with continuous shaking every quarter of an hour to obtain a better extraction after sealing to avoid the entry of impurities and then filtered by a bored cloth or cotton as it was filtered several times to get rid of the suspended impurities with a rotating funnel and then the solution is placed in the rotary vacuum evaporator device (Vacunm Rotary evaporatory) And at a temperature of 40 -45 C for the purpose of obtaining the active substance , then the extract was kept in airtight glass bottles after recording its weight when it was empty and after placing the extract in it to know the weight of the active substance and then placed in the refrigerator until use.

Preparation of the bacterial suspension

The method (Stahly *et al*., 1991) was used. To prepare the bacterial suspension *B.thuringiensis* .

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.

Results and discussion

Presence of *B. thuringiensis*

100 soil samples were collected from different areas in Babil 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 two methods that were used to isolate bacteria from soil samples to choose the best method for isolation. 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 more selective in isolating bacteria, as the number of samples that contained *B. thuringiensis* bacteria when using this method was 57 samples out of the total samples that were examined from the total samples.

Table (1) shows 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*	% percentage	Number of models checked	number of samples that contained *B.thuringiensis*	% Percentage
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.

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)

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**

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 (5). Determining the percentage of crystalline forms present in the local 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 local isolates.

Crystalline protein shape	Total number of isolates	percentage%
Spherical	**17**	**29.8**
Spherical and cube	**13**	**22.8**
Cubic	**10**	**17.5**

Oval	**10**	**17.5**
Bipyramidal	**7**	**12.2**
Total	**57**	

Effect of garlic extracts on the life of the larval stages of the Great Waxworm

The results of the current study concluded that garlic extracts 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 garlic extracts on the destruction of the larvae of the Great Waxworm after 48 hours.

Table (4) The effect of using aqueous and alcoholic extract of garlic on the killing of the larvae of the Great Waxworm after 48 hours, mg/hour

concentration mg/L -1	**aqueous extract**		**alcoholic extract**		**average %**
	direct spray %	**mix with food%**	**direct spray %**	**mix with food%**	
Control	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
1000	**86.66**	**76.6**	**66**	**80**	**77.3**
2000	**20**	**93.3**	**76.6**	**86.6**	**84.1**
3000	**96.66**	**93.3**	**96.6**	**90**	**94.1**
average	**91.1**	**84.4**	**79.7**	**85.5**	**----------**
LSD values Concentration 8.62* , extract 8.62* , Interference 15.36*					
P ≤0.05					

Table (5) The effect of using aqueous and alcoholic extract of garlic on the death of the larvae of the Great Waxworm after 76 hours, mg/hour

concentration mg/L $^{-1}$	**aqueous extract**		**alcoholic extract**		**average %**
	direct spray %	**mix with food%**	**direct spray %**	**mix with food%**	
Control	0.0	0.0	0.0	0.0	0.0
1000	80	80	80	80	80
2000	85.5	83.3	83.3	83.3	83.8
3000	96.6	93.3	96.6	93.3	94.9
average	87.3	85.5	86.6	85.5	----

LSD values Concentration 7.71* NS, extract 7.71* , Interference 13.89*
NS P ≤0.05

Table (6) The effect of using aqueous and alcoholic extract of garlic on the destruction of the larvae of the Great Waxworm after a week of mg/hour

concentration mg/L $^{-1}$	aqueous extract		alcoholic extract		average %
	direct spray %	mix with food%	direct spray %	mix with food%	
Control	0.0	0.0	0.0	0.0	0.0
1000	83.3	70	76.6	63.3	74
2000	85.5	73.3	80	66	76.3
3000	90	76.6	83.3	70	79.9
average	86.2	73.3	79.9	66.6	----
LSD values Concentration 55.7* NS, extract 7.55NS * , Interference 13.62*					
P ≤0.05					

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 in following tables .

Table (6) Effect of different concentrations of bacterial suspension in the destruction of the Great Waxworm larvae after 48 hours (hour/concentration).

Concentration mg/L -1	direct spray %	mix with food%	Average %
Control	0.0	0.0	0.0
^{2}-10	96.6	70.0	83.3
^{5}-10	76.6	63.3	69.9
^{6}-10	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.

Table (7) Effect of different concentrations of bacterial suspension in the destruction of the Great Waxworm larvae after 76 hours. Hour/concentration .

Concentration mg/L -1	direct spray %	mix with food%	Average %
Control	0.0	0.0	0.0
^{2}-10	86.6	83.3	84.95
^{5}-10	73.3	70.0	71.65
^{6}-10	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 (8) The effect of different concentrations of bacteria in the destruction of the the Great Waxworm larvae after one week/hour/concentration.

Concentration mg/L -1	direct spray %	mix with food%	Average %
Control	0.0	0.0	0.0
^{2}-10	90.0	80.0	85.0
^{5}-10	83.3	70.0	76.6
^{6}-10	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 Bt 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,

Discussions

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 by Deluccae *et al*. , (1981) 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 (the first 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 Type *B.thuringiensis*. A study conducted by Iriarte *et al.,* (1998)

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 Apaydin, (2004) in Turkey and the researcher Obeidat *et al.,* (2008) 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 Jamil, (2007) in Iraq and Keshavarzi, that the bi-pyramidal form is the most prevalent.

In a study conducted by the researcher Khairy *et al*. (2011), the results showed the effect of garlic aqueous extract and cypermethrin used by spraying on hard ticks in vitro and in vivo, that a concentration of 452 mg/L of garlic aqueous extract was the most effective concentration in the effect on ticks. Whereas, Cypermethrin at a concentration of 1 ml/L of water achieved faster results with a significant difference at the probability level (0.05). 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 Al-Alan *et al*., (2018) conducting 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.

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- Garlic extracts 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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