Biological Effect of Different Concentrations of Bacillus Thurgnensis Isolated From The Soils of Sawa Lake, Al Muthanna Governorate on The of Hypera postica at Different Time Periods

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Abstract

This study conduct in Al-Muthanna governorate to assess five concentrations of Bacillus thurngensisagonist Hyperapostica. The results showed the presence of Bacillus thurngensisin all the studied sites of Lake Sawa in Muthanna Governorate, and the rates of its presence were close to the same sites, and the rate of its presence in those sites was 35%, and its highest rate was recorded in the north and east of the lake, as it reached 40% and the lowest amounted to 30% in the two sites south and west site. The results of the study showed that five concentrations were taken from bacterial isolates of B. thurngensisdiffered in the rates of killing larvae, pupae and adults of the insect Hyperapostica in vitro, Where the concentrate 1.7 x 10^3 spore/ml was characterized by a higher killing ratecompared to the four studied concentrations, the killing rates were (75.750, 71.080, 69.79, 64.361, 49.117 and 42.060)% for the first larval instar, the second larval instar, the third larval instar, the fourth larval instar, and the pupae, respectively, and the lowest killing rate recorded 6.873% at concentration 0.64 x 10^3 after 24 hours of treatment.

Keywords: Alfalfa Weevil, Hyperapostica, Bacillus thurngensis, Muthanna.

1. Introduction

The most common chemical pesticides in controlling insect pests as a major factor in reducing the quantity and quality of human food, and its role in stopping the damage of agricultural pests cannot be cancelled. It is known that pesticides cause damage and risks to the health of humans and their domestic animals, and the imbalance they cause in the natural balance due to the presence of vital enemies of pests. Therefore, researchers directed towards the use of biological resistance elements to protect the product from the toxic residues of the chemical compounds used.

Bacillus thurngensisin is one of the most important biological resistance factors that has shown high efficiency and gained wide attention [1] and commercial preparations have been made from it that are produced in two forms, the first of which consists of crystalline protein and the second of spores and crystalline protein, and the first formula is the safest B. t. bacteria preparations. With careful specialization in pest control and low production costs and does not leave harmful residues in the soil and water and does not harm the vital enemies of pests because of its precise specialization in the effect and has no effect on contact, as well as non-toxic to farm animals and reptiles [2].

B. thurngensisbeing a gram-positive aerobic bacteria, they are rod-shaped and characterized by the presence of spores and produce toxic proteins called crystals, which are toxic proteins to insects and have been used in the control of various insect orders [3], Ishiata isolated this bacterium for the first time in 1901 in Japan as a cause of sotto disease on the larvae of infected silkworms Bombyx mori, and it acquired the scientific name B. thurngensisin 1915. It continued to be isolated from infected insects belonging to different insect orders [4]. Its production began as commercial preparations in 1970 and marketed with more than 100 commercial preparations [5]. These bacteria spread all over the world [6] as they were found in the soil feeding on the organic remains of dead organisms [7] and isolated from various media, including humus in forests, insects of stored materials, poor soils and deserts, and different agricultural soils. Here’ll was the first to use B. thurngensisin the biological control of insects in the year 1914, it was recorded that more than 50% from the arthropods pest of rice [8]. These
bacteria have a wide range of pathogenicity to many organisms, including protozoa, nematodes, mites, insects, and others [9], and *Hyperapostica* is one of the most important insect pests of the (Coleoptera order: Curculionidae).

Herbst is the first to record this insect in 1784 [10, 11] and it infected some leguminous plants, especially the *Medicago sativa* L. in Britain, Italy, France, Turkey and others. Larvae and adults of the insect create great damage when severely infested, as they feed on leaves and cause the death of infected plants [12]. The success achieved by the pesticides in reducing the numbers of insect encouraged the belief that there are great possibilities for eradicating the insect and limiting its spread [13]. Due to the problems of using pesticides, resorting to other alternatives to control insects has become the main goal of researchers, including the use of pathogenic organisms. The *B. thurmgensis*One of the most common pathogens that can cause epidemics in insect hosts [11] and due to the lack of studies in Iraq on the isolation of *B. thurmgensis*From the soil of Lake Sawa in Al-Muthanna and its use in controlling the economically important Alfalfa Weevil in theeinfected fields. The aims of this study to assess different concentrations of *B. thurmgensis*against *Hyperapostica*.

2. Materials and Methods

2.1 Insect collection and identification

*Hyperapostica* larvae and adults were collected from some cultivated fields in Samawah in southern Iraq and transferred to the laboratory of the Badia Studies Center and Sawa Lake, Al-Muthanna University. The insect was bred on the *Medicago sativa* crop to obtain the adults of the insect and then it saved and sent to the Iraqi Natural History Museum - University of Baghdad for the purpose of confirming its diagnosis.

2.2 Preparing the colony of *H. postica*

For the purpose of preparing an insect colony, the young leaves of *H. postica* were collected from the fields referred to in the previous paragraph and placed in plastic containers with a diameter of 5 cm and a height of 7.5 cm, and isolating each of the roles of the insect separately and with three replications for each treatment, and the leaves of the plant most affected as a result of feeding the insect were replaced whenever needed. The life cycle of the insect was studied in the laboratory at a constant temperature of 25 ± 2 °C using an incubator and in its base glass containers filled with water with a diameter of 19.5 cm and a height of 3.5 cm were placed and 30 g of KOH was dissolved in it in 100 ml of water to obtain a constant relative humidity (70 ± 5%). The thermohygrometer was adopted to ensure the stability of the temperature and relative humidity in it, and the incubator was provided with a 20 W light source with a timer to give a fixed light duration of 16 hours of light and 8 hours of darkness. The incubator was used in the same conditions in all subsequent laboratory experiments.

The eggs of the insect were collected from the places where they were laid by the adults and they were placed 24 hours after being laid by the female in white cylindrical and transparent plastic cups with dimensions 5 x 7.5 cm and placed inside the cups a filter paper moistened with water to provide the moisture necessary for the eggs to hatch. The cups were covered with a mesh cloth and tied with a rubber rope to prevent hatched larvae emerge, the larvae were transferred to the special containers for rearing as indicated previously, and the pupae were isolated upon completion of the aforementioned containers separately and with three replications for each treatment. When the adults emerged, each pair (male and female) of the newly emerged adult insects were placed in the containers prepared for breeding and with three replicates and prepared for study. Coming [14].

2.3 Isolation *B. thurmgensis* from the soil

Taken 40 samples were from the soil of Sawa Lake from four sides (north, south, east and west of the lake) at a distance of 1-15 m from the water of the lake, with 10 samples for each direction, so that the amount of 100 grams of soil was taken at a depth of 15-20 cm after removing the surface layer 5 cm deep, the samples were kept in paper bags until they were transferred to the laboratory [15], where they were sifted well and left to air dry until the process of isolating the mentioned bacteria from them. Bacteria were isolated according to the method which included transferring 1 g of each soil sample to a sterile 250 ml glass beaker containing 10 ml sterile water and placing the beaker on a vibrator for 10 minutes, then withdrawing 1 ml of the suspension (dilution). 10⁻¹ He was placed in a tube and placed in an Eppendorf tube and heat-treated in a water bath at 70 °C for 30 minutes to get rid of the non-spore-forming species. The suspension was cooled and a series of dilutions was conducted for it using sterile water until a dilution was obtained 10⁻². Five dilutions were used (10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶ and 10⁻⁷) where 100 μl of each dilution was transferred to a bacterial suspension containing Nutrient agar and distributed well using a curved glass rod until it dried middle surface, the dishes were kept inverted in the incubator at a temperature of 30 °C for 72 hours, after which the colonies were examined under the microscope after staining with Basic fuchsin dye. A series of biological tests were performed to confirm the presence of crystalline protein and spores or the sporophyte shape characteristic of *B. thurmgensis* Colonies were purified and kept in the
refrigerator until use in bio sterilization, the method of Thiery and Fracchon [16], was adopted to calculate the number of spores of the five concentrations of the aforementioned bacterial suspension, and the Pourplating process was used in this method and successive decimal dilutions were prepared where the test tubes (size 20 cm3) were sterilized with the used pipettes (Prept) so that they contained all Tube on 9 ml of sterile water, 1 ml of the bacterial suspension of the first concentration was added by the first pipette to the first test tube, as its volume became 10 ml, which represents the first dilution ($10^1$), and then 1 ml was withdrawn from the first test tube by means of a pipette to put it in the second test tube, and the volume became 10 ml, which represents the dilution The second ($10^2$) and so on until we reach the seventh dilution ($10^7$) by continuing to draw until the seventh and last tube, withdraw 3 ml of the first dilution ($10^1$) and distribute it to five petri dishes of 14 cm diameter, each containing Nutrient agar medium at a rate of 1 ml for each dish until we reach the seventh dilution ($10^7$). The dishes were incubated at 30 ± 2 ºC for 24 hours. The dishes were taken after the growth of the bacterial colonies according to the five studied dilutions and the other dilutions were neglected, according to the average of the colonies in each Apply and then extract the number of bacteria according to the following equation:[17]

$$\text{Number of bacteria} = \frac{\text{Average number of colonies} \times \text{five plates} \times \text{inverted dilution}}{\text{Sample volume}}$$

The ratio and proportion method was adopted to obtain the concentration of the solution containing $(1.7 \times 10^3)$ spores/ml, and according to the number of cells (spores) in the same way in the other mixed concentrations, we obtained the following concentrations $(1.7 \times 10^3, 1.3 \times 10^4, 1.1 \times 10^5, 0.72 \times 10^6$ and $0.64 \times 10^7$) Spore/ml for $B. thuragensis$ bacteriaother mitigations were neglected. For the purpose of biological sterilization of isolates of bacteria against $H.postica$ larva, pupae and adults in five dilutions $(1.7 \times 10^3, 1.3 \times 10^4, 1.1 \times 10^5, 0.72 \times 10^6$ and $0.64 \times 10^7$) spores/ml which were prepared in the previous paragraph, the uninfected $Medicago sativa$ leaves were sprayed after washing well with sterile water and prepared for feeding the larvae and adults of the insect individually with a 1 liter sprayer at a rate of 3 packages for each of the previously mentioned packages with a comparative treatment in which the plant was sprayed with sterile water only and 10 individuals of the insect larvae, pupae and adults For each container, each of the prepared containers, each containing young leaves of the insect host and circulating in the concentrations mentioned previously, in addition to the comparison treatment, cover the top of each container with a transparent mesh cloth and secure it with a rubber band.

The samples were examined and the percentages of the number of larvae, pupae and adults of the studied insect were recorded. After 24, 36, 48, 72 and 96 hours, the consumed $Medicago sativa$ leaves were replaced with other young leaves continuously after spraying them with pelleted laboratory concentrations. The experiment was conducted in the laboratory and under the conditions referred to previously, and the percentage of insect larval mortality was corrected according to [18].

$$\text{Corrected fatality percentage} = \frac{\% \text{of deaths in the treatment} - \% \text{of deaths in comparison}}{100 - \% \text{of fatalities in comparison}} \times 100$$

The percentages of fatalities were converted into correct values for inclusion in the statistical analysis.

2.4 Statistical analysis

The percent of insect mortality was calculated using the corrected Abbot’s formula. The data were analyzed using analysis of variance (ANOVA), where significant differences between the treatments were observed. Mean values were significantly separated by using the least significant difference (LSD) test at the 5% level.

3. Results and Discussion

3.1 Insect diagnosis

The diagnosis of the insect was confirmed by Prof. Dr. Kazem Saleh Hussein (Department of Biology / College of Science / University of Basra), as follows: (Gyllenhal) (Coleoptera :Curculionidae) $Hypera postica$

3.2 Presence of $B. thuragensis$ in the tested samples

The results showed the presence of $B. thuragensis$. In all soil samples collected from Lake Sawa, however, the number of isolates differed slightly, as 10 samples were examined from each site for the four studied sides of the lake’s soil, as the highest percentage of bacterial presence was recorded 40% in the tested soil samples that were brought from the north and east of the lake., while the lowest was 30% in the south and west of the lake, as shown in Table (1).
Table 1. The presence of *B. thuringiensis* in the studied sites according to the proportions.

| Location relative to the lake | Number of samples taken | Number of samples containing B.t. | Percentage of presence of B.t. |
|------------------------------|-------------------------|----------------------------------|-------------------------------|
| North                        | 10                      | 4                                | 40.0                          |
| South                        | 10                      | 3                                | 30.0                          |
| East                         | 10                      | 4                                | 40.0                          |
| West                         | 10                      | 3                                | 30.0                          |
| Total                        | 41                      | 14                               | 35.0                          |

Prevalence of *B. thuringiensis* in all studied sites 35.0

3.3 Effect of different concentrations of *B. thuringiensis* on the destruction of incomplete roles and *H. postica* adults at different time intervals (hour)

3.3.1 First larval instar

It was found that the different concentrations of bacteria at different time periods had a significant effect on the percentage of deaths of the first larval stage of the insect, and it is clear from Table (2) that the highest percentage of fatalities reached 75.750% at bacteria concentration $1.7 \times 10^3$ for the time period 96 hours, and the lowest percentage was 37.343% in Concentration $0.64 \times 10^{-7}$ at 24 hour exposure. Significant differences were found for the concentrations of bacteria tested at different time periods as well as the overlap, while no significant differences were recorded in the percentage of deaths of the first larval stage of the insect when exposed to a concentration of $0.64 \times 10^{-7}$ for the periods 72 and 96 hours. In this field, it was between Govindaragan et al. 1979 An *B.t.* 100% killing rates of cotton leafworm larvae were achieved after 6 days of treatment in the laboratory, *B.t.* It caused a killing rate of 82.5-95% of the first instar larvae of the potato tuber moth *P. operculella*.

Table 2. Effect of different concentrations of *B. thuringiensis* on the destruction of the first larval stage of *H. postica* at different time periods (hour).

| Bacterial concentration (spore/ml) | Percentage of mortality / hour |
|-----------------------------------|-------------------------------|
|                                   | 24   | 48   | 72   | 96   |
| $1.7 \times 10^3$                 | 52.216 | 64.873 | 70.760 | 75.750 |
| $1.31 \times 10^4$               | 49.903 | 62.050 | 46.105 | 73.993 |
| $1.1 \times 10^5$                | 46.650 | 57.770 | 67.810 | 72.010 |
| $0.72 \times 10^6$               | 41.926 | 54.716 | 65.897 | 69.846 |
| $0.64 \times 10^{-7}$            | 37.343 | 43.033 | 51.680 | 51.606 |

L.S.D for Concentrations (P ≤ 0.05) 1.348
L.S.D for interference L.S.D (P ≤ 0.05) 2.696

3.3.2 Second larval instar

The results of the study showed that the different concentrations of the tested bacteria and within different time periods had a significant effect on the mortality rates for the second larval stage of the insect, and its highest rate was 71.080% at concentration $1.7 \times 10^3$ in the exposure period of 96 hours, and it decreased to its lowest rate of 31.623% in concentration $0.64 \times 10^{-7}$ at exposure for 24 hours (Table 3). **Table 3. The effect of different concentrations of *B. thuringiensis* on the destruction of the second larval stage of *H. postica* at different time periods (hour).**

| Bacterial concentration (spore/ml) | Percentage of mortality / hour |
|-----------------------------------|-------------------------------|
|                                   | 24   | 48   | 72   | 96   |
| $1.7 \times 10^3$                 | 48.143 | 63.290 | 68.633 | 71.080 |
| $1.31 \times 10^4$               | 45.955 | 58.356 | 65.503 | 70.413 |
| $1.1 \times 10^5$                | 40.296 | 49.673 | 62.487 | 68.773 |
| $0.72 \times 10^6$               | 37.21 | 42.290 | 57.613 | 63.826 |
| $0.64 \times 10^{-7}$            | 31.623 | 35.533 | 39.570 | 61.330 |

L.S.D for Concentrations (P ≤ 0.05) 0.477
L.S.D for interference L.S.D (P ≤ 0.05) 1.170
3.3.3 Third larval instar

The different concentrations of bacteria tested in different periods of time had a clear effect on the percentage of deaths of the third larval stage. It reached the highest rate of 69.796% at the period of 96 hours and the lowest at 29.933% at the period of 24 hours. The results of the statistical analysis showed the existence of highly significant differences between the percentage rates of deaths the third larval stage of the insect at a single concentration of bacteria when the insect was exposed to different periods of time as well as within different concentrations at the same time period (Table 4).

Table 4. The effect of different concentrations of B. thurongensis on the destruction of the third larval stage of H. postica at different time periods (hour).

| Bacterial concentration (spore/ml) | Percentage of mortality / hour |
|-----------------------------------|--------------------------------|
|                                   | 24   | 48   | 72   | 96   |
| 1.7×10⁻³                          | 46.326 | 61.340 | 66.053 | 69.796 |
| 1.31×10⁻⁴                         | 44.183 | 56.456 | 63.636 | 69.186 |
| 1.1×10⁻⁵                          | 38.640 | 47.190 | 60.320 | 67.276 |
| 0.72×10⁻⁶                         | 35.810 | 39.900 | 55.076 | 62.236 |
| 0.64×10⁻⁷                         | 29.933 | 32.990 | 37.900 | 59.216 |

L.S.D for Concentrations (P ≤ 0.05) 0.429
L.S.D for interference
L.S.D (P ≤ 0.05) 0.858

3.3.4 Fourth larval instar

The results of the study indicated that there were significant differences in the percentages of deaths of the fourth larval stage of the insect at different concentrations and time periods. It is noted from Table (5) that the highest percentage of fatalities reached 69.363% at concentration 1.7 x 10⁻³ in a period of 96 hours and decreased to 28.280% at 24 hours.

Table 5. The effect of different concentrations of B. thurongensis on the destruction of the fourth larval stage of H. postica at different time periods (hour).

| Bacterial concentration (spore/ml) | Percentage of mortality / hour |
|-----------------------------------|--------------------------------|
|                                   | 24   | 48   | 72   | 96   |
| 1.7×10⁻³                          | 45.583 | 60.670 | 65.526 | 69.363 |
| 1.31×10⁻⁴                         | 43.543 | 54.836 | 63.626 | 68.693 |
| 1.1×10⁻⁵                          | 37.453 | 46.460 | 59.236 | 65.936 |
| 0.72×10⁻⁶                         | 34.313 | 39.960 | 54.026 | 60.946 |
| 0.64×10⁻⁷                         | 28.280 | 31.796 | 36.530 | 58.786 |

L.S.D for Concentrations 0.554
L.S.D (P ≤ 0.05)
L.S.D for interference 1.108
L.S.D (P ≤ 0.05)

3.3.5 Pupae stage

Table (6) showed that there were no significant differences between concentrations 1.7 x 10⁻³ and 1.31 x 10⁻⁴ at 24 hours, as well as the interaction between concentrations 1.31 x 10⁻⁴ and 0.72 x 10⁻⁶ at 48 and 72 hours and concentrations 0.72 x 10⁻⁶ and 0.64 x 10⁻⁷ at the two periods 24 and 48 hours, while high significant differences were found for the other treatments.

3.3.6 Adult stage

The concentrations of bacteria B. thurongensis different at different time periods have less effect on the percentage of mortality of adult insects compared to the four larval stages of the same insect. It was found that the highest mortality rate of the tested insect bugs was 42.060% at concentration 1.7 x 10⁻³ at 96 hours and decreased to 6.873% at concentration 0.64 x 10⁻⁷ at the time of 24 hours (Table 7). The results of the same table also indicated that there were significant differences in the percentage of adult mortality among the studied time periods within the same concentration, except for the concentration 0.64 x 10⁻⁷ in the two periods 24 and 48 hours, and with regard to the significant differences within the same time period and the different concentrations, no differences were observed. Significant over the 24 hour period, while the results showed
significant differences between the concentrations 1.31x10^4 and 1.1x10^5, 0.72x10^6 and 0.64x10^7 at the 24 hour period, and no significant differences were observed in the percentages of adult insect mortality at the concentrations 1.7 x 10^3, 1.31 x 10^4 and 1.1 x 10^5 at the 72-hour period, while differences were found between these three concentrations and the two concentrations 0.72 x 10^6 and 0.64 x 10^7 at the 72-hour period.

No significant differences were recorded between the concentrations 1.7 x 10^3, 1.31 x 10^4, as well as between 1.31 x 10^4 and 1.1 x 10^5, 0.72 x 10^6 and 0.64 x 10^7 at the 96-hour period, while there were differences between concentrations 0.72 x 10^6 and 0.64 x 10^7, as it is noted from the same table that there are no significant differences between concentration 1.31 x 10^4 at the 96-hour period and concentration 1.7 x 10^3 at the 72 hour period.

### Table 6. The effect of different concentrations of *B. thurngensis* on the destruction of the pupae of *H. postica* at different time periods (hour).

| Bacterial concentration (spore/ml) | Percentage of mortality / hour |
|-----------------------------------|-----------------------------|
|                                   | 24  | 48  | 72  | 96  |
| 1.7x10^3                          | 23.39 | 39.00 | 44.926 | 49.117 |
| 1.31x10^4                         | 23.183 | 35.00 | 42.060 | 48.273 |
| 1.1x10^5                          | 19.043 | 28.000 | 41.006 | 46.906 |
| 0.72x10^6                         | 15.490 | 21.000 | 35.580 | 43.333 |
| 0.64x10^7                         | 12.063 | 15.166 | 20.510 | 32.873 |
| L.S.D for Concentrations          | 0.524 |
| L.S.D (P ≤ 0.05)                  | 1.048 |
| L.S.D for interference            |     |
| L.S.D (P ≤ 0.05)                  |     |

### Table 7. The effect of different concentrations of *B. thurngensis* on the destruction of the adults of *H. postica* at different time periods (hour).

| Bacterial concentration (spore/ml) | Percentage of mortality / hour |
|-----------------------------------|-----------------------------|
|                                   | 24  | 48  | 72  | 96  |
| 1.7x10^3                          | 17.496 | 28.726 | 34.253 | 42.060 |
| 1.31x10^4                         | 16.146 | 26.113 | 32.330 | 41.623 |
| 1.1x10^5                          | 12.640 | 20.723 | 31.946 | 38.570 |
| 0.72x10^6                         | 9.243 | 15.166 | 25.990 | 33.976 |
| 0.64x10^7                         | 6.873 | 9.710 | 14.320 | 28.853 |
| L.S.D for Concentrations          | 4.695 |
| L.S.D (P ≤ 0.05)                  | 9.390 |
| L.S.D for interference            |     |
| L.S.D (P ≤ 0.05)                  |     |

The results of the study showed that the concentration of 1.7x10^3 spor/mol of the tested bacteria was superior in killing the four roles of *H. postica* compared to other concentrations of the same bacteria. It was found that the modern larval ages of the insect were the most sensitive to the concentrations used against it compared with the third and fourth larval instars as well as the pupal and adult and this is consistent with what [3] indicated in their study on the effect of doses of *B. thurngensis, kurstaki* against the Mediterranean flour moth *Ephestia kuehniella*, and the results of the study indicated the slow development and small size of *H. postica* larvae treated with bacteria, which survived the destruction, as well as an increase in the average duration of their development [19].

### Conclusion

The study also showed that the larvae and adults of the insect *H. postica* treated with bacteria stopped feeding, diarrhea appeared, paralyzed their movement and ended with death, the symptoms of infection were also delayed in the appearance of some infected insects, and their movement and feeding were slow, and their body became soft and tends to brown, especially the last movements of the body of the larva and the adult. This lethal effect of the tested bacteria on *H. postica* may be due to two types of toxins produced by bacteria, and the first type includes a major toxin known as Beta-exotoxin, which includes the toxins Flytoxin and Thuringiensin, and is considered toxic to invertebrates, so it cannot be entered into biological control, and this is consistent with what [3] mentioned, the same researcher also indicated that the bacteria B.t. It produces a second type of toxin known as Endotoxin which is known as ICP (Insecticidal Crystal Protein), and [8] showed that insects treated with bacteria that produce these toxins begin to ingest the toxic crystals referred to, as they dissolve in the alkaline medium of the insect’s midgut, and the protease enzyme converts them into effective toxins represented by highly toxic polypeptides.
with a high molecular weight of 55-70 kDa that encapsulate the receptors. The specialized cells lining the midgut membrane of insects infected with bacteria degrade these cells and eventually the insect stops feeding and dies of starvation [6].

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