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# Efficiency of Local Entomopathogenic Bacterial Isolates against the Citrus Leafminer, *Phyllocnistis citrella*

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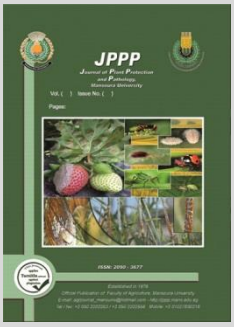


## ABSTRACT

Citrus is one of the most important fruit crops in the world. It is infested with the citrus leafminer (CLM), *Phyllocnistis citrella* Stainton (Lepidoptera: Gracilleridae) which is a significant pest affecting citrus plants in nurseries and orchards. The present study aimed firstly at isolating the bacteria that naturally correlated with CLM larvae and then evaluating the pathogenicity and toxic effect of these isolates against CLM larvae in comparison with the commercial product of *Bacillus thuringiensis* (Portecto), and finally identifying the most effective isolate. During the present study, eight different bacterial slants (slants from 1 to 8) were isolated from the collected larvae of CLM which exhibited abnormal symptoms. At the highest concentration (100% of the initial suspension), the mortality percentage of CLM larvae (2nd instar) after six days of treatment was 100±0.0% with the usage of slant 4 followed by slant 3 (96.6±3.3%), and then both of Protecto and slant 6 (93.3%). In the control treatment, there no mortality was detected. Statistical analysis showed that mortality percentages significantly increased by both elapsed time and the concentration used. The toxicity of the tested slants showed that the most toxic slants were 4, Protecto, and slant 3; where the values of $LC_{90}$ were 49.67, 61.31, and 108.99 x $10^6$ cfu/ml, respectively. Therefore, the bacteria of slants 4 and 3 showed higher mortality percentages in comparison with the other isolated bacterial slants. Slant 4 identified as *Bacillus rugosus*; while slant 3 identified as *Priestia megaterium*.

***Keywords:*** Bacillus, Citrus, $LC_{90}$, Protecto, Slant

## INTRODUCTION

Citrus is one of the most important fruit crops worldwide, with a production of more than 100 million tons annually. Citrus fruits are of great importance to the economic and social development of producing countries worldwide. They constitute export products and processing into various derivatives such as juices, jams, and essences, as they can be a source of employment (Loussert, 1989; Khechna, 2011; Khechna *et al*., 2017 and Mahmoudi *et al*., 2017).

The citrus leafminer (CLM), *Phyllocnistis citrella* Stainton (Lepidoptera: Gracilleridae) is a significant pest affecting citrus trees in nurseries and orchards. Where, the larvae mine and form serpentine-like appearance on the leaves, tender twigs, and fruits. The affected leaves start to curl and dry upon infestation. It is native to subtropical and tropical Asia (CAB, 1986) and established itself as a major pest of citrus throughout the Middle East (Moreira et al., 2006), where Egypt is located. In Egypt, it was detected in 1994; then, it spread rapidly throughout most of the citrus growing areas to attack many citrus orchards and nurseries (Hashem, 1996; Abo-Sheaesha, 1997; Jacas *et al*., 1997; Eid, 1998 and El-Afify *et al*., 2018). It attacks more than half of the newly formed leaves of citrus trees (Wilson, 1991). Severe infestations can retard the growth of young new growths and may affect the production of mature plants (Grafton-Cardwell *et al*., 2008 and Dileepkumar *et al*., 2022).

The control of agricultural pests is a constant concern owing to the economic damage and environmental impact of non-biological practices. Producing fruits with zero or little pesticide residue is crucial to satisfying the demands of importing countries. So, bio-rational insecticides are well suited for use in organic food production and play a much greater role in the production of pesticide-free food (Isman, 2006). In Egypt, interest in the use of bio-rational pesticides is increasing, which depend on natural materials such as plants, animals, microbes, and mineral derivatives, for the control of insect pests. Recently, the use of bio-rational insecticides (any type of natural or synthetic material proven effective against pest populations) has very much increased. *Bacillus thuringiensis* subsp. *krustaki* is a soil bacterial strain and it is widely used for controlling larval populations of lepidopterous insects; where it is safe for many non-target insects with minimal environmental impacts (Lacey *et al*., 2001). These isolated bacteria induced higher mortality in their original insect hosts than in natural concentrations. Therefore, the search for new microbial agents for pest control is one of the most promising needs in the field of biological control. Accordingly, the isolation of more local entomopathogens that would be more adapted to the local pest strains and possess greater insecticidal activities or broader host range (Abd-Elazim *et al*., 1991; Osman, 1992, Keller, 1998 and El-Metwally *et al*., 2010).

From the previous review, this research aims to isolate the bacteria that naturally correlated with CLM larvae, to evaluate the pathogenicity and toxic effects of bacterial isolates in comparison with the commercial product of *B. thuringiensis* (Portecto) against CLM larvae, and to identify the most effective isolate(s).

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## MATERIALS AND METHODS

During the present study, the pathogenicity and toxic effect of the isolated local bacteria against the second larval instar of CLM (because the response to insecticides is higher at this instar) was evaluated in comparison with the commercial product of *B. thuringiensis* (Portecto) under laboratory conditions. Portecto was obtained from the Plant Protection Research Institute, Agricultural Research Center, Ministry of Agriculture.

**1. Isolation of bacterial isolates:**

At Sherbeen district (located in Dakahlia governorate, Egypt), an area of about 15 feddan (1 feddan equal to 4200 $m^2$) cultivated with navel orange was selected for the present study. Periodic samples were collected weekly over a whole year (from 20/9/2021 till 25/9/2022). Each sample consisted of 100 young leaves were randomly collected from five trees and different directions of each tree (north, south, east, west, and center) with a rate of 5 leaves/direction. The leaves were kept in polyethylene bags, transferred to the laboratory, and examined under binocular microscope.

To isolate bacterial agents from CLM larvae, living and dead individuals from the collected samples that exhibited abnormal symptoms were distinguished and put into sterilized tubes. These tubes were transferred to the Microbiological lab, Microbiology Department, Faculty of Agriculture, Mansoura University.

The dilution plate method was used for the isolation of the insect microorganisms. The insect was crushed, and then sterile water was added. Aliquots of 1 ml from a sterilized water suspension containing (larvae and plant leaf) were transferred to Petri-dishes, and then nutrient agar medium (OXOID) was added and mixed thoroughly thereafter, bacterial colonies were picked after three days of incubation at 30 °C, after that the colonies were picked and maintained in stock on nutrient agar slopes for further studies.

**Nutrient agar medium (Skerman, 1967):**

It consisted of (g/L): peptone, 5.0; beef extract, 3.0; water 1000 ml; agar 15; pH, 7.0.

**2. Pathogenicity and toxic effect of natural and commercial bacteria against CLM larvae:**

**Bacterial inoculant preparation:**

The bacterial growths, of the naturally isolated bacteria, on the nutrient agar slants were scraped, using 5 ml sterile tap water, and then transferred to a flask containing 50 ml sterile nutrient broth, bacterial isolates were grown for 2 days at 30 °C. Afterward, the density of each slant suspension was counted and recorded as cfu/ml. A series of concentrations (five concentrations) of 50 ml slant suspension were prepared as 5, 10, 25, 50, and 100% of the initial suspension (the highest one) and Protecto (Table, 1).

**Table 1. Spore concentrations (as cfu/ml) of bacterial isolates (slants from 1 to 8) after growing for 2 days at 30 °C in addition to the used concentrations of Protecto.**

| Bacterial isolates | Concentrations | | | | |
|---|---|---|---|---|---|
| | 5% | 10% | 25% | 50% | 100% |
| Slant 1 | $6.5x10^6$ | $13.0x10^6$ | $32.5x10^6$ | $65.0x10^6$ | $130.0x10^6$ |
| Slant 2 | $7.75x10^6$ | $15.5x10^6$ | $38.75x10^6$ | $77.5x10^6$ | $155.0x10^6$ |
| Slant 3 | $5.25x10^6$ | $10.5x10^6$ | $26.25x10^6$ | $52.5x10^6$ | $105.0x10^6$ |
| Slant 4 | $6.0x10^6$ | $12.0x10^6$ | $30.0x10^6$ | $60.0x10^6$ | $120.0x10^6$ |
| Slant 5 | $6.25x10^6$ | $12.5x10^6$ | $31.25x10^6$ | $62.5x10^6$ | $125.0x10^6$ |
| Slant 6 | $7.0x10^6$ | $14.0x10^6$ | $35.0x10^6$ | $70.0x10^6$ | $140.0x10^6$ |
| Slant 7 | $5.4x10^6$ | $10.8x10^6$ | $27.0x10^6$ | $54.0x10^6$ | $108.0x10^6$ |
| Slant 8 | $5.5x10^6$ | $11.0x10^6$ | $27.5x10^6$ | $55.0x10^6$ | $110.0x10^6$ |
| Portecto | $2.5x10^6$ | $5.0x10^6$ | $10.0x10^6$ | $25.0x10^6$ | $50.0x10^6$ |

Then, the bacterial concentrations were used against CLM larvae. The same concentrations were prepared from the commercial bacterial product (Portecto).

**Bioassay:**

The bioassay test was applied using the leaf-dip technique which was described by Amiri-Besheli (2008). Only leaves with actively feeding 2nd instar larvae were completely excised from the petioles from navel orange trees. To keep leaves turgescent during the bioassay, each petiole was covered with wet cotton. Leaves were dipped individually, for approximately 10 sec. into each suspension, air-dried for approximately 2 hours, and placed at the bottom of plastic Petri dishes (9 cm in diameter) which were previously lined with wet filter paper. The experiment for each concentration of each bacterium was replicated three times; where, each replicate included 10 larvae, along with a control group. Leaves used for control treatment were treated with sterile nutrient broth mixed with sterile tap water. All Petri dishes were incubated at 25±1°C and 80±5% RH with a 16:8 h (L:D) photoperiod. After 3, 4, 5, and 6 days post-treatment, the numbers of living and dead larvae in each replicate were counted under a stereomicroscope and recorded.

**3. Identification of bacterial isolates:**

Bacterial isolates were identified by Sigma Scientific Services Co. (located on the 6th of October city, Egypt), using a 16S rRNA sequence. The sequences obtained were then compared with the existing sequences in the NCBI database. The MEGA 11.0 software was used for multiple sequence alignment of 16S rRNA sequences. The accession numbers were obtained from the NCBI GenBank database for 16S rRNA sequences of bacteria.

**4. Statistical analysis:**

Mortality percentages of the treated larvae by the evaluated bacterial isolates were corrected by Abbot's formula (Abbot, 1925). The results of the bioassay test were subjected to analysis of variance (One-way ANOVA) and calculated the standard error (SE) and the least significant differences (L.S.D.) by using CoHort software (CoHort, 2004). Lethal concentrations ($LC_{50}$ and $LC_{90}$), and slope values were calculated by the Finney method using LDP-line software (Finney, 1971). The toxicity index was calculated according to the Sun equation (Sun, 1950).

## RESULTS AND DISCUSSION

**Results**

During the present study, eight different bacterial slants were isolated from the collected larvae of CLM which showed abnormal symptoms. All of the isolated slants were evaluated against CLM larvae to identify which of these isolates acts as a biocontrol agent against the pest or not in comparison with the commercial product of *Bacillus thriengensis* (Protecto).

Data illustrated in Table (2) show that after three days of treatment, mortality percentages were higher with the usage of the highest concentration (100%) of slant 6 (53.3±3.3%) followed by slant 4 (43.3±3.3%), slant 3 (40.0±5.7%) and slant 1 (40.0±5.8%). The mortality percentages in CLM larvae by the rest of the treatments (whether slants or concentration) were less than 40.0%. Protecto and control treatments showed no effect on CLM larvae after 3 days of treatment. Statistical analysis showed

that there were significant differences between concentrations in the same slant and between all treatments whether between slants or concentrations in addition to the control treatment (where general LSD was 9.5***).

**Table 2. Mortality percentages of CLM larvae caused by five concentrations of the bacterial isolates in comparison with Protecto after 3 days of treatment under laboratory conditions.**

| Bacterial isolates | Concentration% of the initial suspensions | | | | |
|---|---|---|---|---|---|
| | 5 | 10 | 25 | 50 | 100 |
| Slant 1 | 13.3±3.3c | 23.3±3.3bc | 33.3±3.3ab | 30.0±5.8b | 40.0±5.8a |
| Slant 2 | 0.0±0.0 b | 0.0 ±0.0b | 0.0 ±0.0b | 0.0±0.0b | 6.6±3.3a |
| Slant 3 | 0.0 ±0.0 c | 3.3±3.3c | 6.6 ±3.3b | 13.3±3.3ab | 40.0±5.7a |
| Slant 4 | 3.3 ±3.3c | 13.3 ±3.33c | 26.6 ±6.6b | 36.6±3.3ab | 43.3±3.3a |
| Slant 5 | 3.3 ±3.3c | 10.0 ±10.0bc | 20.0 ±5.7abc | 26.6±8.8ab | 33.3±3.3a |
| Slant 6 | 10.0 ±0.0c | 16.6 ±3.3bc | 26.6 ±6.6bc | 33.3±8.8b | 53.3±3.3a |
| Slant 7 | 3.3 ±3.3c | 16.6 ±6.6ab | 20.0 ±5.7a | 23.3±3.3a | 26.6±3.3a |
| Slant 8 | 0.0±0.0b | 0.0 ±0.0b | 0.0 ±0.0b | 26.6±8.8a | 26.6±8.8a |
| Portecto | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 | 0.0±0.0 |
| Control | | | 0.0±0.0 | | |
| General LSD | | | 9.5*** | | |

**Means bearing the same small letters in each raw are not significantly different at 0.05 probabilitly level.**

After four days of treatment, mortality percentages were higher at the highest concentrations of slants 6, 4, 3, and 1 where mortality percentages were 93.3±3.3, 83.3±3.3, 63.3±3.3 and 60.0±5.7%, respectively (Table, 3). Concerning Protecto, it showed the highest effect (mortality of 50.0±5.8%) when it was used in its highest concentration. At all of the tested treatments (except slant 2), mortality percentages decreased significantly as the spore concentration declined. There was no mortality in the control treatment was detected. Statistical analysis showed that there were significant differences between all treatments either between slants or between concentrations.

**Table 3. Mortality percentages of CLM larvae caused by five concentrations of the bacterial isolates in comparison with Protecto after 4 days of treatment under laboratory conditions.**

| Bacterial isolates | Concentration% of the initial suspensions | | | | |
|---|---|---|---|---|---|
| | 5 | 10 | 25 | 50 | 100 |
| Slant 1 | 23.3±3.3c | 40.0±5.7bc | 46.6±8.8ab | 60.6±5.7a | 60.0±5.7a |
| Slant 2 | 20.0±3.3a | 20.0±0.0a | 20.0±3.3a | 23.3±3.3a | 33.3±8.8a |
| Slant 3 | 16.6±3.3b | 20.0±0.0b | 23.3±3.3b | 60.0±5.7a | 63.3±3.3a |
| Slant 4 | 13.3±3.3d | 46.6±8.8c | 56.6±6.6bc | 73.3±3.3ab | 83.3±3.3a |
| Slant 5 | 16.6±6.6b | 26.6±8.8b | 30.0±5.7b | 33.3±6.6ab | 56.6±8.8a |
| Slant 6 | 30.0±5.7c | 43.3±13.3bc | 56.6±8.8bc | 66.6±8.8ab | 93.3±3.3a |
| Slant 7 | 6.6±3.3c | 33.3±8.8a | 36.3±3.3a | 40.0±5.7a | 43.3±8.2a |
| Slant 8 | 0.0±0.0c | 13.3±3.3bc | 30.0±11.5ab | 43.3±8.8a | 46.6±8.8a |
| Portecto | 13.3±3.3c | 26.6±6.8bc | 33.3±3.3b | 40.0±5.8ab | 50.0±5.8a |
| Control | | | 0.0±0.0 | | |
| General LSD | | | 14.1*** | | |

**Means bearing the same small letters in each raw are not significantly different at 0.05 probabilitly level.**

As shown in Table (4), mortality percentages after five days of treatment were equal to or higher than 90% when CLM larvae treated with the highest concentrations of Protecto (90.0±5.7%), slant 6 (93.3±3.3%), slants 3 & 4 (96.6±3.3%) in addition to 50% concentration of slant 4 (90.0±5.7%). Statistical analysis showed that there was a significant decrease in mortality percentages by the decrease of treatment concentration (except that of slant 8), also there were significant differences between all treatments whether between slants or concentrations. There was no mortality detected in the control treatment.

**Table 4. Mortality percentages of CLM larvae caused by five concentrations of the bacterial isolates in comparison with Protecto after 5 days of treatment under laboratory conditions.**

| Bacterial isolates | Concentration% of the initial suspensions | | | | |
|---|---|---|---|---|---|
| | 5 | 10 | 25 | 50 | 100 |
| Slant 1 | 23.3±3.3c | 40.0±5.7bc | 46.6±8.8ab | 60.0±5.7ab | 63.3±8.8a |
| Slant 2 | 20.0±5.7c | 26.6±3.3bc | 30.0±5.7bc | 40.0±8.8a | 53.3±8.8a |
| Slant 3 | 16.6±3.3d | 36.6±3.3c | 50.0±5.7c | 70.0±5.7b | 96.6±3.3a |
| Slant 4 | 13.3±3.3c | 60.0±3.3b | 66.6±5.7b | 90.0±5.7a | 96.6±3.3a |
| Slant 5 | 23.3±8.8b | 26.6±6.6b | 33.3±8.8b | 36.6±3.3b | 66.6±8.8a |
| Slant 6 | 36.6±3.3d | 53.3±8.8cd | 66.6±8.8bc | 76.6±8.8ab | 93.3±3.3a |
| Slant 7 | 13.3±3.3b | 36.6±6.6a | 36.6±6.6a | 40.0±5.7a | 50.0±5.7a |
| Slant 8 | 46.6±3.3b | 53.3±8.8b | 56.6±8.8ab | 63.3±8.8ab | 76.6±3.0a |
| Portecto | 33.3±6.6c | 50.0±0.0bc | 60.0±5.8b | 63.3±8.8b | 90.0±5.7a |
| Control | | | 0.0±0.0 | | |
| General LSD | | | 15.6*** | | |

**Means bearing the same small letters in each raw are not significantly different at 0.05 probabilitly level.**

The accumulative mortality all over the successive 6 days is illustrated in Table (5). At the highest concentration (100% of the initial suspensions), the mortality percentage of CLM larvae was higher (100±0.0%) with the usage of slant 4 followed by slant 3 (96.6±3.3%). Mortality percentage reached 93.3% when larvae were treated with Protecto or slant 6. While in control treatment there were no mortality percentages. Statistical analysis showed that there were significant differences between most concentrations in the same treatment and between all treatments whether between treatments or concentrations.

**Table 5. Mortality percentages of CLM larvae caused by five concentrations of the bacterial isolates in comparison with Protecto after 6 days of treatment under laboratory conditions.**

| Bacterial isolates | Concentration% of the initial suspensions | | | | |
|---|---|---|---|---|---|
| | 5 | 10 | 25 | 50 | 100 |
| Slant 1 | 26.3±3.3c | 40.0±5.7bc | 46±3.3ab | 60.0±3.3a | 63.3±3.3a |
| Slant 2 | 20.0±5.7b | 26.6±3.3b | 30.0±5.7b | 36.6±5.7ab | 53.3±8.8a |
| Slant 3 | 16.6±3.3d | 36.6±3.3c | 50.0±5.7c | 70.0±5.7b | 96.6±3.3a |
| Slant 4 | 13.3±3.3c | 60.0±5.7b | 73.3±6.6b | 90.0±5.7a | 100±0.0a |
| Slant 5 | 23.3±3.3b | 26.6±8.8b | 33.3±8.8b | 36.6±3.3b | 66.6±8.8a |
| Slant 6 | 36.6±0.0d | 53.3±8.8cd | 66.6±8.8bc | 76.6±8.8ab | 93.3±3.3a |
| Slant 7 | 33.3±3.3c | 46.6±8.8bc | 56.6±8.8abc | 63.3±8.8ab | 73.3±3.3a |
| Slant 8 | 46.6±8.8b | 56.6±8.8ab | 56.6±8.8ab | 63.3±8.8ab | 76.6±6.6a |
| Portecto | 56.6±8.8b | 63.3±3.3b | 73.3±8.8ab | 76.6±3.3ab | 93.3±6.7a |
| Control | | | 0.0±0.0 | | |
| General LSD | | | 15.4*** | | |

**Means bearing the same small letters in each raw are not significantly different at 0.05 probabilitly level.**

Statistical analysis showed that there were significant differences between inspections when Slant 3 and Protecto were used against CLM larvae at all of the tested concentrations (Table, 6); where, mortality percentages were increased by the increase of elapsed days (Tables, 2-5). Concerning slants 4 and 2, there were significant increases in mortality percentages by the elapsed time (inspections) at all of the tested concentrations except at the concentration of 5%; in which there were no significant differences between inspections (Table, 6). Slant 5 showed no significant differences between inspections when it was used against CLM larvae at all of the tested concentrations.

**Table 6. The least significant differences between inspections (3, 4, 5, and 6 days of treatment) refer to mortality percentages of CLM larvae caused by the tested concentrations of the bacterial isolates and Protecto under laboratory conditions.**

| Bacteria | Concentration% of the initial suspensions | | | | |
|---|---|---|---|---|---|
| | 5 | 10 | 25 | 50 | 100 |
| Slant 1 | 10.8$^{ns}$ | 17.1$^{ns}$ | 21.7$^{ns}$ | 17.1** | 15.3* |
| Slant 2 | 16.3$^{ns}$ | 12.1** | 13.3** | 18.0** | 25.4** |
| Slant 3 | 9.4** | 13.3** | 15.3*** | 17.1*** | 13.3*** |
| Slant 4 | 10.8$^{ns}$ | 20.3** | 23.6** | 15.3*** | 9.4*** |
| Slant 5 | 19.5$^{ns}$ | 28.2$^{ns}$ | 23.06$^{ns}$ | 19.5$^{ns}$ | 25.4$^{ns}$ |
| Slant 6 | 12.1** | 30.2$^{ns}$ | 27.1* | 28.7* | 10.8*** |
| Slant 7 | 10.8*** | 25.4$^{ns}$ | 24.9$^{ns}$ | 20.3* | 18.8*** |
| Slant 8 | 15.3*** | 21.0*** | 22.4*** | 28.7$^{ns}$ | 23.6** |
| Portecto | 18.8*** | 12.1*** | 18.0*** | 18.0*** | 17.1*** |

**$^{ns}$: Non-significant at 0.05 probability level, $^{*}$: significant at 0.05 probability level, $^{**}$: significant at 0.01 probability level, $^{***}$: significant at 0.001 probability level.**

Mathematically, there were positively direct relationships between the concentrations of the tested treatments (isolated bacterial slants & Protecto) and the mortality percentages of CLM larvae caused by these treatments. With each increase in the concentration by a unit (1x10$^6$ cfu/ml), the mortality percentage increased by 0.73, 0.58, and 0.68% when using slant 3, slant 4, and Protecto, respectively (which showed the highest responses). For the rest of the other slants, each increase in their concentrations by a unit (1x10$^6$ cfu/ml) led to an increase in the mortality percentages by values ranging between 0.21 and 0.37% (Table, 7).

**Table 7. Linear relationships and coefficient of determination ($R^2$) between concentrations of the tested treatments (bacterial slants & Protecto as x10$^6$ cfu/ml) and mortality percentages of CLM larvae under laboratory conditions.**

| Bacteria | Relationship | $R^2$ |
|---|---|---|
| Slant 1 | Mortality% = 36.95 + 0.26 Concentration | 0.622 |
| Slant 2 | Mortality% = 21.44 + 0.21 Concentration | 0.972 |
| Slant 3 | Mortality% = 24.93 + 0.73 Concentration | 0.929 |
| Slant 4 | Mortality% = 40.80 + 0.58 Concentration | 0.640 |
| Slant 5 | Mortality% = 23.28 + 0.32 Concentration | 0.911 |
| Slant 6 | Mortality% = 45.51 + 0.37 Concentration | 0.868 |
| Slant 7 | Mortality% = 40.98 + 0.33 Concentration | 0.821 |
| Slant 8 | Mortality% = 49.54 + 0.25 Concentration | 0.924 |
| Protecto | Mortality% = 60.00 + 0.68 Concentration | 0.913 |

The results in Table (8) show the toxicity of the tested bacterial slants against second-instar larvae of CLM after 6 days of treatment. According to $LC_{50}$, the most toxic product was Protecto followed by slants of 8, 6, 4, 7, and 3 where the values of $LC_{50}$ were 1.85, 7.98, 13.53, 13.62, 16.98, and 19.99 x 10$^6$ cfu/ml, respectively. Whereas, the most toxic treatment was slant 4, Protecto, slant 3, and slant 6 where the values of $LC_{90}$ were 49.67, 61.31, 108.99, and 148.39 x 10$^6$ cfu/ml, respectively. The $LC_{90}$ of the rest treatments was high.

The toxicity index and relative toxicity at $LC_{90}$ arranged the treatments in two groups as follows: The relatively highest group (in descending order as slant 4, Protecto, and slant 3, respectively) and the relatively lowest group (in descending order as slants 6, 7, 1, 8, 5 and 2, respectively) (Table 8).

**Table 8. Toxicity of bacterial slants against CLM larvae in comparison with Protecto after 6 days of treatment under laboratory conditions.**

| Bacterial slants | $LC_{50}$ (x10$^6$ cfu /ml) | | | $LC_{90}$ (x10$^6$ cfu /ml) | | | Slope | Toxicity index (%) | | Relative Toxicity (Fold) ($LC_{90}$) |
|---|---|---|---|---|---|---|---|---|---|---|
| | $LC_{50}$ | Confidence limits (95%) | | $LC_{90}$ | Confidence limits (95%) | | | LC50 | LC90 | |
| | | Lower | Upper | | lower | Upper | | | | |
| Slant 1 | 38.72 | 27.84 | 56.64 | 1622.58 | 606.48 | 10133.86 | 0.790 | 100 | 3.06 | 10.40 |
| Slant 2 | 175.81 | 101.25 | 520.91 | 16868.69 | 2978.94 | 833999.18 | 0.647 | 23.15 | 0.29 | 1 |
| Slant 3 | 19.99 | 10.09 | 35.94 | 108.99 | 86.97 | 492.89 | 1.740 | 13.66 | 45.57 | 154.78 |
| Slant 4 | 13.62 | 5.98 | 22.99 | 49.67 | 40.34 | 191.34 | 2.281 | 13.56 | 100 | 339.63 |
| Slant 5 | 77.98 | ------ | ------ | 3312.99 | ------- | ------- | 0.787 | 10.88 | 1.50 | 5.09 |
| Slant 6 | 13.53 | 9.91 | 17.26 | 148.39 | 102.36 | 255.71 | 1.232 | 9.24 | 33.47 | 113.68 |
| Slant 7 | 16.98 | 11.17 | 23.87 | 813.98 | 328.80 | 4522.93 | 0.763 | 4.77 | 6.10 | 20.72 |
| Slant 8 | 7.98 | 2.52 | 13.90 | 2237.96 | 503.52 | 117002.45 | 0.524 | 2.37 | 2.22 | 7.54 |
| Portecto | 1.85 | 0.80 | 2.99 | 61.31 | 35.38 | 164.56 | 0.843 | 1.05 | 81.01 | 275.13 |

For slope values (Table 8 and Fig. 1), the steepest toxicity line of slant 4 possessed the highest slope value (2.281) followed by slant 3 (1.740) and slant 6 (1.232); which indicates relatively higher homogeneity of the tested population. Whereas, the flattest line was that of slant 8 possesses the lowest slope value (0.524). The remaining slope values were 0.843 (Protecto), 0.790 (slant 1), 0.787 (slant 5), 0.763 (slant 7) and 0.647 (slant 2).

From the previous results, it can be noticed that the bacteria of slant 4 and slant 3 showed higher mortality percentages compared to the other bacterial slants (Tables, 2-5). Also, mathematical relationships between the concentrations and the resulting mortality percentages showed that the highest responses were obtained when slant 4 and slant 3 were used (Table, 7). These findings were supported by LDP-line software; which showed that $LC_{90}$ of the isolated slants was the highest when slant 4 and slant 3 were used (Table 8 and Fig. 1). Therefore, slants 4 and 3 were selected for identification in Sigma Scientific Services Company.

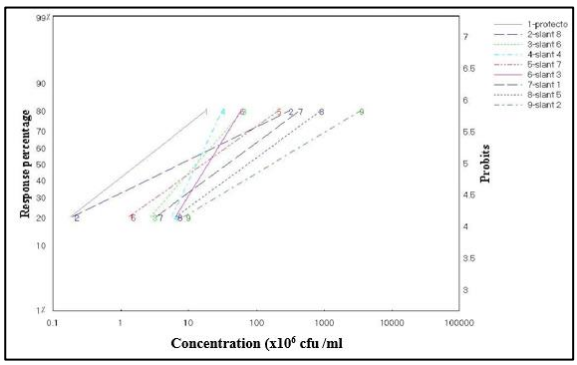


**Fig. 1. Regression lines of the bacterial slants against CLM larvae compared to Protecto after 6 days of treatment under laboratory conditions.**

Slant 4 (Fig. 2) was identified as *Bacillus rugosus* (Bhattacharya *et al*., 2020); while slant 3 (Fig 3) was identified as *Priestia megaterium* (formerly known as *Bacillus megaterium*) (Gupta *et al*., 2020).

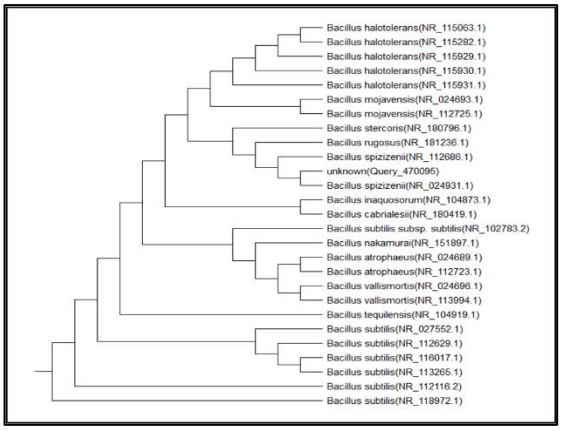


**Fig. 2. NCBI GenBank database for 16S rRNA sequences of bacteria identified Slant 4 as *Bacillus rugosus***

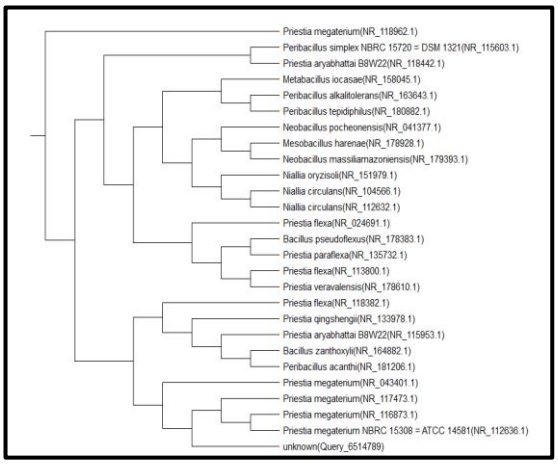


**Fig. 3. NCBI GenBank database for 16S rRNA sequences of bacteria identified Slant 3 as *Priestia megaterium***

## Discussion

The unconscious use of insecticides has led to many adverse effects on the environment. These adverse effects of the chemical insecticides that are applied against insect pests warrant the development of strategies that could reduce the usage of insecticides for controlling insect pests. On the other hand, the effect of insecticides in citrus orchards against the CLM is difficult to achieve the maximum CLM larval control and it is not very effective since the larvae are fed in mines inside the plant leaf. This may be attributed to the overlapping generations of CLM and its larvae are protected by a cuticular layer of the leaves in the serpentine mine, in addition, the pupal stage is also protected by the rolled leaf margins (Raga *et al*., 2001). Therefore, biological control agents may be the alternative tool for controlling insect pests; where, the biological control paradigm changed after the potential of entomopathogenic bacteria was discovered, especially species belonging to the genus *Bacillus* (Glare and O'Callagan, 2000).

Laboratory bioassay indicated that the local isolations of *B. rugosus* and *P. megaterium* (formerly known as *Bacillus megaterium*) act as effective entomopathogenic bacterial isolates against CLM larvae, indicating that they are valuable candidates to control this pest. These isolates were so high, as it was found that they were more effective than Protecto (the commercial product of *B. thuringiensis*) since suspensions of *B. rugosus* and *P. megaterium* caused larval mortality to reach 100±0.0 and 96.6±3.3%, respectively after 6 days of treatment; while Protecto caused larval mortality of 93.3±6.7%. Shapiro *et al*. (1998), Khyami-Horani and Ateyyat (2002), and Moustafa (2004) recorded that *Bacillus* spp. exhibited high activity against CLM populations. Various studies have been done on the insecticidal influences of *B. megaterium* (Padgham and Sikora, 2007; Aksoy and Ozman-Sullivan, 2008; Huang *et al*., 2010). Khyami-Horani *et al*. (1999) reported that *B. megaterium* was highly toxic to larvae of *Culiseta longiareolata* (Diptera: Culicidae). Aksoy and Ozman-Sullivan (2008) reported that isolates of *B. megaterium* were successfully used for *Aphis pomi* (Hemiptera: Sternorrhyncha: Aphididae) causing 92% to 100% mortality within five days of the treatment. According to Aksoy *et al.* (2018), *B. megateium* may be possible to use the SAkc-2 as a potential biocontrol agent against *Palomena prasina* L. (Heteroptera: Pentatomidae) in quite large hazelnut plantations of Turkey.

For the reason of their capacity to produce toxins during sporulation, *Bacillus* species are applied as alternative biocontrol agents (Pietrantonio *et al*., 1993; Zhang *et al*., 1995; Wagner *et al*., 1996). Shapiro *et al*. (1998) and Dias *et al.* (2005) demonstrated that *B. thuringiensis* can penetrate the larval mine and kill the larvae inside. Polanczyk *et al*. (2000) determined the mortality percentages caused by different strains of *B. thuringiensis* against the second-instar larvae of *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) were similar and suggested that these results could be related to the composition of crystals and their toxic effects. Similar findings were observed by Follas (1995) in a study with two different strains of *B. thuringiensis* against lepidopteran pests.

The obtained data showed that mortality percentages caused by the most effective slants (*B. rugosus* and *P. megaterium*) and *B. thuringiensis* (Protecto) increased significantly with the increase in elapsed time. These findings are in agreement with Saeidi and Saeidi (2016); who found that the efficiency of *B. thuringiensis* against CLM larvae increased as the elapsed time increased. On the other hand, the efficiency of *B. rugosus*, *P. megaterium* (as entomopathogenic agents for CLM larvae), and *B. thuringiensis* (Protecto) increased significantly with the increase of their concentrations. These results came in the same line of Shapiro *et al*. (1998), Khyami-Horani and Ateyyat (2002), and Saeidi and Saeidi (2016); they found that the mortality rates of CLM larvae increased with the increase in *B. thuringiensis* concentrations. This may be attributed to the low production of proteinase by *B. thuringiensis* (Saeidi and Saeidi, 2016). Also, these results are consistent with those obtained by Beattie and Hardy (2004); they found that a low concentration of *B. thuringiensis* caused low mortality to *Diaprepes abbreviates* (Coleoptera: Curculionidae).

Based on these results, we recommend that the identified bacteria (*Bacillus rugosus* and *Priestia megaterium*) may be a promising approach to control CLM, but further studies will be required in the future, especially field studies

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# فاعلية العزلات البكتيرية الممرضة المحلية ضد نافقة اوراق الموالح

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## الملخص

تعتبر الموالح واحدة من أهم محاصيل الفاكهة في العالم. وهي تصاب بنافقة أوراق الموالح والتي تعتبر افه هامة تؤثر علي نباتات الموالح في المشاتل والبساتين. تهدف الدراسة الحالية أولاً إلى عزل البكتيريا التي ترتبط بشكل طبيعي بيرقات نافقة أوراق الموالح، ثم تقييم التأثير الممرض والسام لتلك العزلات البكتيرية ضد يرقات نافقة أوراق الموالح ومقارنة ذلك مع المنتج التجاري لبكتيريا الـ *Bacillus thuringiensis* (البرتيكتو)، وأخيراً تحديد العزلة الأكثر فاعلية ضد الافة. خلال الدراسة الحالية تم عزل ثمانية سلالات بكتيرية مختلفة (السلالات من ١ إلى ٨) من يرقات نافقة أوراق الموالح والتي أظهرت أعراضًا غير طبيعية علي اليرقات. اوضحت النتائج أنه عند التركيز الأعلي (١٠٠٪ من المعلق الأولي)، وصلت نسبة الوفيات في يرقات العمر الثاني لنافقة أوراق الموالح إلي ١٠٠٪ بأستخدام السلالة ٤ ، ثم حققت السلالة ٣ نسبة موت ٩٦,٦ %، ثم حققت كل من السلالة ٦ ومركب البروتيكتو ٩٣,٣٪ نسبة موت. كما لم يتم ملاحظة اي نسب موت في معاملة الكنترول. أوضح التحليل الاحصائي أن نسب الموت إزدات بصورة معنوية مع زيادة تركيز المعاملة والوقت المنقضي. كما وضحت اختبار سمية السلالات المختبرة أن السلالات الأكثر سمية كانت السلالة ٤ والبروتيكتو والسلالة ٣ ، في حين أن قيم التركيزات المسببة لـ ٩٠٪ موت في التعداد كانت ٤٩,٦٧ و ٦١,٣١ و ١٠٨,٩٩ X10^٦ جرثومة /ملي علي التوالي. ولذلك أوضحت العزلات البكتيرية ٤ و ٣ نسب موت عالية مقارنة بالعزلات البكتيرية الاخري. كما تم تعريف السلالة ٤ ب *Bacillus rugosus* بينما تم تعريف السلالة ٣ ب *Priestia megaterium*.