Research Article

Evaluation of Cell-Free Supernatant from *Xenorhabdus nematophila* Bacteria and Two Insecticides against Mealybug *Phenacoccus solenopsis* under Laboratory and Field Conditions

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Abstract | Mealybug *Phenacoccus solenopsis* is one of a group of highly aggressive and invasive insect pests. Leaving large numbers of *P. solenopsis* on host plants can cause significant economic losses and damage. The effectiveness of commercially available chemical insecticides and biopesticides against cotton mealybugs was evaluated under lab and field conditions by using the secreted toxins of symbiotic bacteria *Xenorahbdus nematophlia*, Blaiser® as a biopesticide, and chemical pesticide Sivanto®. Our results indicate that the efficacy of secreted toxins by symbiotic bacteria *X. nematophlia* was the highest after the chemical pesticide Sivanto® and superior to the biopesticide Blaiser®, as the effectiveness recorded was (98.72%), while it was (100%) and (93.3%) for the chemical and biological pesticides, respectively, during the test period of 24-72 hours. The LC50 values after 48 and 72 hours of spraying free cell suspension of *X. nematophila* bacteria were 29.178 and 17.788. In contrast, the LC50 values for Blaiser® were 28.118 and 25.907, respectively. The more toxicity increased with the increase in the exposure period compared to the chemical compound Sivanto®. The results confirmed that the use of a free cell suspension of *X. nematophlia* bacteria gave excellent and promising results in controlling the mealybug *P. solenopsis*, comparable to and sometimes superior to pesticides of chemical origin.

Received | September 15, 2024; **Accepted** | October 10, 2024; **Published** | October 28, 2024
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Citation | Jabbar, A.S. and S. Murad. 2024. Evaluation of cell-free supernatant from *Xenorhabdus nematophila* bacteria and two insecticides against mealybug *Phenacoccus solenopsis* under laboratory and field conditions. *Sarhad Journal of Agriculture,* 40(4): 1322-1329.
DOI | https://dx.doi.org/10.17582/journal.sja/2024/40.4.1322.1329
Keywords | Mealybug *Phenacoccus solenopsis*, *Xenorahbdus nematophlia*, Bacteria, Free cell suspension, Biopesticide, Insecticides

Introduction

Mealybug *Phenacoccus solenopsis* (Pseudococcidae; Hemiptera) (CMB) is an invasive pest of many crops, spreading onto at least 213 species of host plants belonging to 56 plant families, with a preference for Asteraceae, Solanaceae, Malvaceae, and Fabaceae (Ben-Dov *et al.*, 2015). Ornamental plants such as *Lantana camara* (Verbeneceae), *Pilea serpyllacea*, and *Portulaca grandifloria* are the preferred hosts for this pest, especially *Hibiscus tiliaceus* (common names include sea hibiscus, beach hibiscus, and coastal hibiscus), which has recently been widely cultivated in home and public gardens in Iraq. *P. solenopsis* causes

damage to plants directly by sucking sap in new growth parts, as well as indirectly by secreting waxy secretions. This limits photosynthesis. Infected plants appear weak and wrinkled. In addition, leaves appeared distorted during early infestation turn yellow and eventually fall off (Waqas *et al.*, 2021). Leaving large numbers of CMBs unchecked can cause significant economic losses. For example, in India and Pakistan, 30–60% of losses in cotton crops were attributed to CMB infestation during 2005–2009 (Dhawan *et al.*, 2007; Nagrare *et al.*, 2009).

The effectiveness of chemical treatment of mealybugs increases their resistance to pesticides with continuous exposure, which kills the pest's natural enemies and has harmful effects on humans. By implementing agro ecosystems where highly destructive broad-spectrum insecticides are reduced and restoring the natural balance of the fauna, we must look for ways to control this insect pest biologically. Among them is the use of the nematode *Steinernema carpocapsia* (Steinernematidae), which contains in its own cyst a non-nutritious infective stage in the third stage (Bird and Akhurst, 1983). It is a bacterium, *Xenorahbdus nematophlia* belonging to the Entrobacteriaceae family, with a symbiotic relationship with the parasitic nematode *S. carpocapsia* on insects. The bacteria enter and carried to the insects by the nematodes, where the nematodes secrete them into the blood of the insects. The bacteria multiply and secrete toxins that kill the insect after 48 hours, digest its contents, convert them into liquid materials that are easy for the nematodes to swallow to feed on them, grow and reproduce inside the body of the targeted insect (Balcerszak, 1991). The study aimed to compare the effects of cells free suspension of the bacterium *X. nematophila* and two commercial pesticides, Sivanto and Blaiser.

Materials and Methods

Chemical insecticides (Sivanto)®

The heterocyclic organic compound flupyradifurone is one of the most effective compounds for exterminating a wide range of insect pests belonging to the Butenolides group, and it is marketed as an alternative to the pesticides Neonicotinoids. It was commercially sold as Sivanto (Nauen *et al.*, 2015).

MoA: Flupyradifurone operates reversibly as an agonist on insect nicotinic acetylcholine receptors, as evidenced by chemical similarity analysis. It has rapid activity on an extensive variety of sucking pests in laboratory conditions.

The chemical insecticide Flupyradifurone was tested using five concentrations of 0, 0.5, 1, 1.5, and 2 ml/L water.

Bio-insecticide

Bio insecticide (Spinosad): Blaiser® is a chemical that belongs to the group Spinosyns. It is a compound extracted from the products of the bacterial fermentation of *Saccharopolyspora spinosa* bacteria. This group of pesticides is very effective in combating many insect pests, including many species of Lepidoptera and Diptera, along with some members of several other insect orders (Thompson *et al.*, 1995; Smith, 2024). Spinosyns have a unique mode of action (MOA), which involves the destruction of nicotinic acetylcholine receptors (Kirst, 2010). The biocide Spinosad, which is available in the commercial Iraqi market under the name BLAISER, was tested using five concentrations: 0, 0.25, 0.50, 0.75 and 1 ml/L of water (El-Kady *et al.*, 2007).

Free cell suspension of X. nematophila

This experiment was designed to check the toxicity of *X. nematophila* cell-free suspension at different concentrations against mealy bugs. The suspensions at five concentrations were selected for application: 0%, 0.25 %, 0.50%, 0.75%, and 100% with one drop of Tween 80 diluted in sterile water 1 ml of suspension was sprinkled on 30 mealy bugs, adults.

Laboratory experiments

Insects: Adult mealybug *P. solenopsis* was collected from a *Hibiscus tiliaceus* Plant nursery at the Agricultural Faculty, Muthanna University, Muthanna, Iraq in March 2024. Leaf discs of 5 cm diameter were cut from the growing apex of the plant *H. tiliaceus* leaves, which were cleaned with distilled water and dried before use. Adult mealybug insects were treated with treatments and carefully moved to 9-cm plates containing two pieces of leaves, which were applied separately and lined with moist filter paper. Each treatment involved 5 mealy bug adults and was replicated six times including controls. Mortality was assessed after 24, 48, and 72 hours of exposure to bio-chemical insecticide. Live and dead insects are detected by touching them gently under a binocular microscope (40X Magnification).

Isolation Xenorhbudus nematophila bacteria

The newly formed infective stage (IJs) of the insect-pathogenic nematode *S. carpocapsae* was taken and placed in a 1.5 ml test tube. The infective stage was washed three times with sterile distilled water, then using 10% sodium hydroxide (NaOH) to sterilize the infective stage using a centrifuge at 8000 cycles to sediment. The infectious phase at the bottom of the test tube was washed twice with distilled water to remove sodium hydroxide residue.

The infectious phase was crushed with sterile non-ionic water using a plastic column prepared for this purpose to fit the bottom of the test tube completely. The suspension was then collected and distributed on a 9 cm Petri plate containing medium. Nutritional NBTA (Nutrient agar, 0.025% bromothymol blue, triphenyltetrazoliumchchloride 0.004% (TTC). The plate was incubated at a temperature of 28±2˚C for 48 hours in dark conditions (Akhurst, 1986), where a pure bacterial culture of *X. nematophila* was obtained. A single colony of purified bacteria *X. nematophila* was selected and inoculated into 500 ml of Nutrient Broth (NB) liquid medium, placed on a shaker at 150 rpm for 3 days at 28 ˚C. The color of the medium will turn from yellow to orange. by using a spectrophotometer adjusted to 600 nm The bacterial cell suspension reached to 1.0 optical density. To obtain concentrations of the free cell bacterial suspension, bacterial culture was centrifuged (8000 rpm for 30 min). A 0.22 m Millipore filter was used to separate the supernatant which was diluted in sterile distilled water.

Felid experiments

Hibiscus plants were grown in 16-cm diameter plastic pots using a commercially available potting mix. Each pot contained one healthy plant. When the plants reached 60–70 cm, each was infested with 10 adult bugs. The plants were placed on large wire-mesh tables capable of holding up to 20 pots.

The insecticide control units were designed as randomized complete blocks or split plots with five replicates and five plants (per experimental unit) within treatments. Three treatments were included: Sivanto, blaiser, and free cell suspensions of *X. nematophlia* bacteria, in addition to the control treatment. All the plants in the design were placed on a single table.

The plants were sprayed approximately two weeks after infestation with the tested insecticides at the dose recommended above (Materials and Methods) using a handheld boom with a single nozzle in the center (above the plant) of the boom, configured to spray horizontally into the plant. The weather conditions at the spraying time ranged from 22 to 35 ˚C and the relative humidity from 18 to 25%. Efficacy evaluations were conducted after insecticides were applied 24 hrs. before spraying and then 24, 48, and 72 hrs. after spraying at different orientations on the same plant. Where were possible, treatment efficacy was determined by counting the number of large (third instars and adult) and small (creeping and second instar) CMB on each leaf and stem of each plant. The total number of small and large CMB per plant was counted as a sum on the leaf and stem and then summed over the entire plant. When counting was not practical or possible, treatment efficacy evaluation was based on a five-point scoring index: scores of 0, 1, 2, 3, and 4 were assigned to 0=0, 1=1- 4, 2=5-9, 4=10-15, 15, and >15 CMB. In GHE 8, large CMB were counted, while small CMB were scored. Score of 4 was assigned a value of 30 CMB, which although somewhat conservative, is well above the range of densities that cause significant damage to vegetation (Khan, 2014).

Data analysis

CoStat version 6.45 program for data manipulation and statistical analysis, was used to analyze the data collected in each experiment using analysis of variance (ANOVA) and the LSD test (Sawyer, 2009). Data in the form of percentages were converted to arcsine values for ANOVA. The LC50 values were estimated using the Ldp Line 1.0 software program. The Toxicity Index was estimated for each bioassay period using the equation below (Sun, 1950):

Toxicity index= LC50 of the most effective compound/ LC50 of the other tested compound × 100

Results and Discussion

In the bioassay test, the data in Tables 1 and 2 indicate that the longer the exposure time, the greater the toxicity, as the LC_{50} value reached (42.479), (35.031), and (14.538) (Figure 1), while the LC_{90} value was (154.509), (102.208), and (61.183) for the pesticides free cell suspensions of *X. nematophlia* bacteria, Blaiser®, and Sivanto®, respectively. The toxicity index of the above-tested compounds against the nymphs of the mealybug after 28 hours of the test recorded a value of 39.139, 40.614, and 100, respectively.

Table 1: *Toxicity compounds under laboratory conditions against nymphs of Phenacoccus solenopsis.*

Toxicity index**	LC_{90} (mg/L)	Slope ± SE	Confidence limits (95% CL)		LC_{50} (mg/L)	Time	Insecticides
34.223	154.509	2.285	48.574	35.922	42.479	24 h.	Free cell suspensions of *X. nematophlia* bacteria
70.823	81.149	2.865	33.346	23.897	28.972	48 h.	
-	69.421	2.232	24.016	10.968	18.501	72 h.	
41.500	102.208	2.756	39.777	29.684	35.031	24 h.	Spinosad (Blaiser)®
92.917	74.08	2.438	27.046	16.14	22.083	48 h.	
-	62.424	2.001	20.064	7.497	14.289	72 h.	
100	61.183	2.053	20.561	6.195	14.538	24 h.	(Sivanto)®
100	79.005	2.189	25.97	13.826	20.519	48 h.	
-	-	-	-	-	-	72 h.	

*** Toxicity index = {LC_{50} for the most effective compound/ LC_{50}}* 100, for each bioassay period.*

Table 2: *Toxicity compounds under laboratory conditions against adults of Phenacoccus solenopsis.*

Index**	LC_{90} (mg/L)	Slope ± SE	Confidence limits (95% CL)		LC_{50} (mg/L)	Time	Insecticides
32.981	142.198	2.539	50.178	38.517	44.473	24 h.	Free cell suspensions of *X. nematophlia* bacteria
39.139	104.801	2.308	34.532	22.754	29.178	48 h.	
-	94.092	1.772	24.28	9.574	17.788	72 h.	
30.048	226.716	1.922	56.874	40.878	48.814	24 h.	Spinosad (Blaiser)®
40.614	97.719	2.369	33.32	21.873	28.118	48 h.	
-	82.586	2.545	30.74	20.111	25.907	72 h.	
100	70.694	1.876	20.737	7.603	14.668	24 h.	(Sivanto)®
100	58.048	1.815	18.063	2.888	11.42	48 h.	
-	-	-	-	-	-	72 h.	

*** Toxicity index = {LC_{50} for the most effective compound / LC_{50}}* 100, for each bioassay period.*

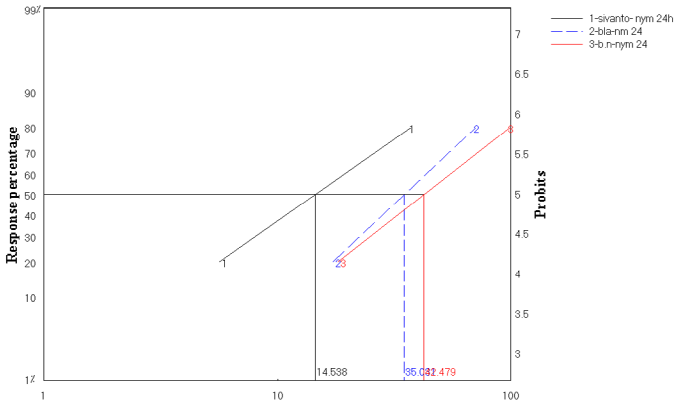

Figure 1: *LC50 value of the tested pesticides after 24 hours of treatment against mealybug nymphs.*

It is clear from the data in Table 1 that spraying with free cell suspensions of *X. nematophlia* bacteria was highly toxic against insect nymphs after 72 hours, and the LC_{50} value reached 17.788 compared to 25.907 of the biocide (Blaiser)®.

The LC_{50} value of the tested compounds against adults after 24 hours of spraying was 44.473 for free cell suspensions of *X. nematophlia* bacteria and 48.814 for the biological compound Blaiser®, compared to 14.668 for the chemical compound Sivanto®, indicating that free cell suspensions of *X. nematophlia* bacteria is more toxic than Blaiser® (Figure 2).

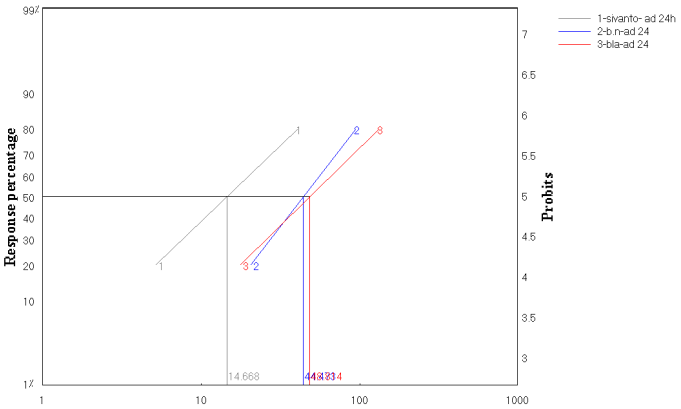

Figure 2: *LC_{50} value of the tested pesticides after 24 hours of treatment against mealybug adults.*

The data in Table 2 showed that the LC_{50} values after 48 and 72 hours of spraying for free cell suspensions of *X. nematophlia* bacteria were 29.178 and 17.788, respectively. In contrast, the LC^{50} values for Blaiser®

Table 3: *Efficacy of biocides 24 to 72 hours after spraying.*

Treatments	Before 24 hrs.	After 24 hrs.	After48 hrs.	After 72 hrs.	L.S.D 0.05	*Index	Efficacy 100%
(Sivanto)®	12.8 a	1.8 b	0 c	0 c	1.31	0	100%
Spinosad (Blaiser)®	11.2 a	4.2 b	2.8 bc	0.75 c	2.41	1	93.3%
Free cell suspensions of *X. nematophlia* bacteria	15.6 a	3 b	0.8 c	0.2 c	1.21	1	98.72%
Control	14.6 a	13.2 a	13.6 a	15.4 a	2.41	3	0

**index: 0 = 0, 1=1- 4, 2=5-9, 4=10-15, 15, and >15.*

were 28.118 and 25.907, respectively. The more toxicity increased with the increase in the exposure period compared to the chemical compound Sivanto®.

The data in Table 3 indicate that the efficacy of free cell suspensions of *X. nematophlia* bacteria was the highest after the chemical pesticide Sivanto® and superior to the biopesticide Blaiser®, as the effectiveness recorded was (98.72%), while it was (100%) and (93.3%) for the chemical and biological pesticides, respectively, during the test period of 24-72 hours. Because the nematode that causes insect diseases, *Steinernema carpocapsia* (Steinernematidae), contains the bacterium *Xenorahbdus nematophlia*, which belongs to the family of symbiotic Entrobacteriaceae responsible for killing insects, it is transmitted to insects by these nematodes, which secrete it into the insect's hemolymph. Then the bacteria multiply and secrete toxins that have the ability to kill the insect within 48 hours, digesting its contents and turning them into liquid substances that are easy for the nematode to swallow to feed, grow, and reproduce inside the body of the target insect (Balcerszak, 1991).

This agrees with (Khush and Lemaitre, 2000), who refer to *X. nematophlia* as responsible for killing insects within 24-48 h by secreted protein CyA; it is an exoenzyme that leads to cytotoxicity.

In this regard, (Hemalatha *et al*., 2018; Singh *et al*., 2023) indicated that the entomopathogenic bacteria *X. nematophlia* produces two phenotypic forms of the cell, phase I and phase II, both of which are usually pathogenic to insects. The defense of insects against entomopathogenic bacteria is dependent on the immune defense system. It is composed of different types of hemocytes. When entomopathogenic bacteria reach the insect hemolymph, the hemocytes aggregate and surround the bacteria to form nodulation (Dunn, 1986).

On the other hand, the bacteria *Xenorhabdus* spp. produces a variety of antibacterial and antifungal compounds, some of which are also active against insects, nematodes, protozoa, and cancer cells. Some compounds, like protein UnA, produced by some strains of *X. nematophila*, prevent the hemocytes of the insect from aggregating and forming capsules or nodules, inhibiting the activity of phenoloxidase, an important enzyme in the insects immune response artillery, according to (Forst *et al*., 1997; Ribeiro *et al*., 1999). Jabbar *et al*. (2024) demonstrated the effectiveness of nematodes *S. carpocapsia* that carrying bacteria *X. nematophlia* against sunn pest *Eurygaster testudneria* achieved lethal time on adults was between 4.01-4.42 days.

Since Mealybug *P. solenopsis* is a group of highly aggressive and invasive insect pests (Seinen *et al*., 2011; Tong *et al*., 2022) indicated that the waxy layer is essential for mealybugs to adapt to different environments and plays an important role in protecting them from insecticide penetration. This waxy layer consists of a complex mixture of lipids, including cuticular hydrocarbons (CHCs), fatty acids, esters, alcohols, and ketones (Ahmad *et al*., 2020). CHCs were found to be the predominant chemical components of the waxy layer of mealybugs. The composition of HCFCs may vary greatly among mealybug species, which means that HCFCs are closely related to the biological functions of the waxy layer (Arunkumar *et al*., 2018). By studying the molecular mechanism responsible for wax synthesis, Tong *et al*. (2022) demonstrated the importance of the *PsFAR* gene, which plays a vital role in wax synthesis and is essential for water retention and protection of insects from insecticide treatment. In other words, if the production of wax filaments is affected, such as by silencing the *PsFAR* gene by RNAi, the mortality rate of mealybugs in response to insecticide treatment is likely to be higher. This also illustrates the importance of proteins produced by *X. nematophlia* and their effect on the *PsFAR* gene responsible for wax synthesis in this mealybug. Thus, increasing the effectiveness

of *X. nematophlia*, causing insects to lose this waxy layer (Figure 3) and expose them to attack by insect parasites. Given the vital protective roles that the wax layer provides against water loss and exposure to toxic substances in the environment (Gibbs, 2007; Ginzel and Blomquist, 2016), the destruction of this wax layer, through methods such as the use of wax-degrading bacteria, has been considered a viable means of managing mealybugs (Salunkhe *et al.*, 2013; Gupta *et al.*, 2022).

Figure 3: *Adult losing waxy layer after exposing to free cell suspensions of X. nematophlia bacteria.*

Conclusions and Recommendations

The results showed that the use of nematodes (*S. carpocapsia*) carrying bacteria (*X. nematophlia*) gave excellent and promising results in controlling the mealybug (*P. solenopsis*), comparable to and sometimes superior to pesticides of chemical origin. In conclusion, the production of proteins by *X. nematophlia* plays a crucial role in the biosynthesis of wax in mealybugs, thus contributing to their adaptation to water loss and stress resulting from pesticides, which enhances the use of biological control in modifying ecosystems and reduces the use of chemical pesticides and their harmful effects on the environment and humans.

Acknowledgements

Authors are grateful to Dr. Jawad B. Al-Zaidawi in the Ministry of Science and Technology, Department of Biocontrol Insect for technical support and cooperation to complete this research work.

Novelty Statement

The study is novel for being the first approach in cell-free supernatant of *X. nematophila* bacteria, promising biopesticides to kill and remove the wax layer from mealybugs.

Author's Contribution

Ahmed Shamkhi Jabbar: Conducted the experiment, review and editing.
Sadoon Murad Sadoon: Data analysis, manuscript write-up.

Conflict of interest

The authors have declared no conflict of interest.

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