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Original article

Larvicidal Activities of Entomopathogenic Nematode *Steinernema carpocapsae* and Zinc Oxide Nanoparticles Against the Black Cutworm, *Agrotis ipsilon*

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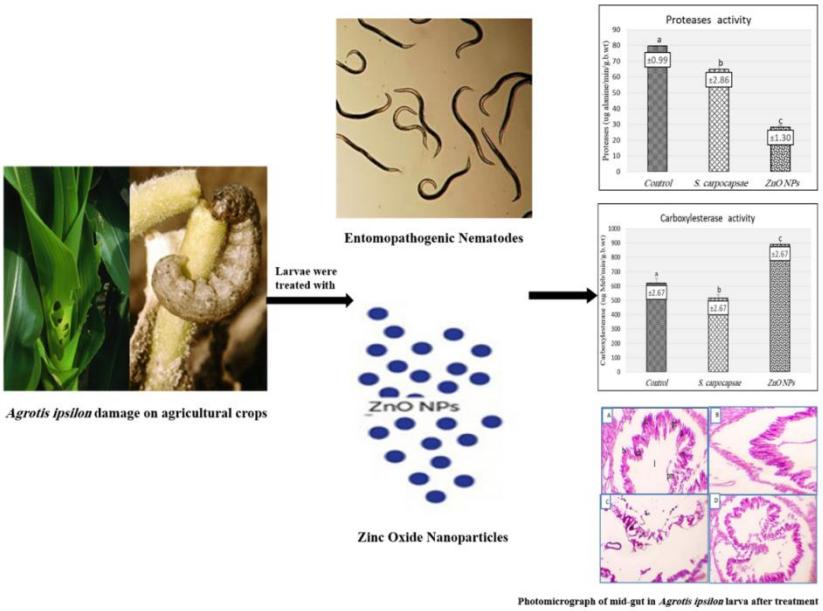
ABSTRACT

The present study was conducted to determine the effect of entomopathogenic nematode *Steinernema carpocapsae* (*S. carpocapsa*) infective juveniles and zinc oxide nanoparticles (ZnO NPs) against *Agrotis ipsilon* larvae. Castor leaves treated with various concentrations of *S. carpocapsae* (20, 40, and 80 IJs/larva) and ZnO NPs (150, 300, and 450 ppm) were fed to the fourth larval instar. Further mortality percentage biochemical assays and some histological studies were carried out to explain the effect of tested larvicides on *A. ipsilon*. The mortality percent of 4th larval instar due to the *S. carpocapsae* and ZnO NPs treatment increased up to 86.67 and 96.67%, respectively after 96 h of application. The median lethal concentration (LC_{50}) of *S. carpocapsa* IJs were 66.93, 38.71, and 25.86 IJs/larva at 48, 72, 96 h post treatment, respectively. The corresponding LC_{50} values of ZnO NPs were 773.42, 396.70, 273.58, and 182.46 ppm at 24, 48, 72, 96 h post treatment, respectively. The results of biochemical assays and histological examination proved the lethal effects of the tested items.

Graphical abstract



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1. Introduction

The black cutworm, *Agrotis ipsilon*, is considered one of the most damaging and disreputable phytophagous pests in Egypt. It attacks many crops, including cotton, fruits, and vegetables **[1].** The larvae feed on the leaf's epidermis, young seedlings, and shoots either beneath or above the ground at night **[2].**

Because of the intensive damage caused by the attack of this pest, it is very necessary to find effective control methods. The arbitrary expenditure of insecticides led to the development of insect resistance and high ecological risk; there are great challenges to find alternative control strategies to reduce the risks to the environment, humans, and non-target organisms **[3, 4]**.

Entomopathogenic nematodes (EPN) and nanoparticles for insect control can provide new strategies against the potentially undesirable environmental impact of organic insecticides to upgrade environmental and human safety and could minimize insect control costs [5-7]. Entomopathogenic nematodes of the genus *Steinernema* are effective control methods against many pest species [8]**.** Infective juveniles (IJs) infest their host body and release bacteria into the hemolymph, causing the speedy death of the insect by septicemia. Subsequently, the EPNs feed on the tissues of dead insects and reproduce themselves in many generations [9]. Several studies have investigated EPNs' virulence to BCW larvae under laboratory conditions (third, fourth, fifth, sixth, seventh instars). Fourth and fifth instars were the most susceptible to the EPN species [10-12].

Nanoparticles are characterized by a molecular size range from 1- 100 nm and high-water solubility and stability of the formulations, if compared to other insecticides. Furthermore, nanoparticles have high efficiency against insects, weeds, and plant pathogens with a high degree of safety to the environment and public health [13, 14]. Zinc oxide (ZnO) is considered one of the significant metal oxides due to its physical and chemical properties. It has great potential in the nanoparticle's biosynthesis for clinical purposes [15].

This work aimed to evaluate the potential of entomopathogenic nematode *S. carpocapsae*, and Zinc oxide nanoparticles (ZnO NPs) against *A. ipsilon* 4th larval instar. As well as study the impact of EPN and ZnO NPs on some physiological aspects of the larvae, such as changes in proteolytic enzyme (protease activity) and detoxifying enzyme (carboxylesterase activity), in addition to some histological studies on the midgut. Finally, the insecticidal efficiency of EPNs and ZnO NPs is compared.

2. Materials and methods

2.1. Experimental insects

The study was conducted at the laboratory of Zoology and Entomology Department, Faculty of Science, Al-Azhar University (Girls' branch), Cairo, Egypt. The eggs of *Agrotis ipsilon* laboratory population were obtained from the Black cutworm Department, Plant Protection Research Institute, Dokki, Giza, Egypt. These eggs were surface sterilized with formalin (10%) Vapor treatment and preserved until hatched [16]. After hatching, the larvae were transferred into a spotless glass container, 1 liter, and tightly covered with gauze. The larvae were supplied daily with fresh *Ricinus communis*, castor leaves. When larvae reached the third larval instar, they were moved to a clean, larger glass jar (2 liters) till the pupal stage. Then, we transferred the pupae into a jar with paper towels on the bottom and covered with muslin till the adult's emergence. Afterward, the adults were transferred to larger jars containing a piece of cotton moistened with 20% sugar solution (changed every 2 days) and covered with black gauze strips for egg deposition. The eggs were collected daily, transferred to new jars, and kept until hatching [17]. The rearing technique was performed under experimental conditions of 25 ± 2 ° C and 65 ± 5% R.H.

2.2. Tested materials

Entomopathogenic nematodes

In the study, the entomopathogenic nematode Steinernema carpocapsae was obtained from the Bioinsecticide Unit of the Plant Protection Institute, Agriculture Research Center, Giza, Egypt. The nematodes were reared for three generations in the 4th larval instar of the black cutworm *A. ipsilon* according to [18]. The emerging infective juveniles (IJs) were harvested from white traps and stored in distilled water at 4°C to test the nematodes. The nematodes were placed at room temperature for at least one hour before use.

Zinc oxide (ZnO) nanoparticles synthesis:

Zinc oxide nanoparticles components were obtained from Naqaa Company, Cairo, Egypt, and were synthesized according to [19] by the precipitation method using zinc acetate and sodium hydroxide (NaOH) as precursors. ZnO nanoparticles were produced by mixing aqueous solutions of zinc acetate and sodium hydroxide. In the typical procedure, 5 g of zinc acetate was dissolved in deionized water. 2M NaOH was added dropwise under magnetic stirring.

After the addition was complete, the stirring continued for 30 min at 60 degrees. The precipitates were washed with pure water several times. Then the obtained precipitates were dried for 2hrs. Three different concentrations (150, 300 and 450 ppm) of the tested ZnO NPs were prepared from the prepared stock solution (5000ppm) by diluting with distilled water in volumetric flasks to give the necessary concentrations [20].

Characterization of ZnO nanoparticles:

For nanoparticles morphology and size determination, transmission electron microscopy (TEM) is a powerful method for detecting morphology and size determination. Nanoparticles were observed at voltage of 180 KV using AMT camera unit at Electron Microscopy Unit (The Regional Center for Mycology and Biotechnology, Al-Azhar University, Egypt). Diluted colloidal ZnO NPs solution was deposited on carbon coated-copper grid and left to dry at room temperature. TEM images of the ZnO NPs that deposited on the grid were captured for morphological evaluation [21].

2.3. Bioassay

Susceptibility of A. ipsilon larvae to S. carpocapsa

The susceptibility assays were adopted using three concentrations of the infective juveniles. Fifteen plastic cups of 13-cm diameter were provided by 200g of sterilized soil. The soil in each cup was moistened with 20 ml of sterile distilled water. Groups of five cups were used for each concentration. Five individuals of *A. ipsilon* 4th larval instar were put in each cup. Each cup was uniformly pipetted with 2ml of the tested nematode concentrations (100, 200, and 400 IJs, representing 20,40, and 80 IJs per larva, respectively. Five control cups were treated with tap water to obtain equal numbers of controlled and treated larvae. All larvae were provided with castor leaves as a source of food. All cups were labeled and incubated under laboratory conditions (25 ± 2 °C and 65±5 % R.H). Mortality percentage was calculated after 24, 48, 72, and 96 h post infection PI. The lethal effect of IJs was recorded by counting dead insect larvae. On the other side, live treated larvae were stored for histological and biochemical assay [1].

Larvicidal activity of zinc oxide nanoparticles

The fourth larval instar was treated with ZnO NPs; as they have a high feeding consumption. Fourth larval instars were fed on castor leaves treated with different concentrations of ZnO NPs (150, 300, and 450ppm). Three replications were done with 10 larvae per replicate. Castor leaves were dipped for a few seconds in each tested concentration and in distilled water for the control, then allowed to dry for a few minutes before being placed in the corresponding cups. Ten starved larvae were put in each cup and allowed to feed. The accumulated mortalities were recorded at 24, 48, 72, and 96 h post-treatment [22]**.** The lethal effect of ZnO NPs concentration was recorded by counting dead insect larvae. On the other side, live treated larvae were stored for histological and biochemical assays.

2.4. Biochemical assay

Sample preparation

Biochemical assays were estimated after 24h post-exposure to the LC_{50} of the tested larvicides to determine the level of proteolytic enzyme (protease activity) and detoxifying enzyme (carboxylesterase activity). Three replicates per treatment with 20 4th live larval instars for each enzyme were set up. The larvae were homogenized in distilled water (50 mg/ 1ml). Homogenates were centrifuged at 8000 rpm for 15 min at 5oC in a refrigerated centrifuge. The final supernatants were kept frozen until used [23]**.**

Apparatus

For biochemical analysis, larvae were homogenized in a chilled glass Teflon homogenizer (ST-Mechanic-Preczyina, Poland). Then, centrifugation was done by a cooling centrifuge (6 MR, USA). A double-beam ultraviolet/ visible spectrophotometer (Spectronic 1201, Milton Roy Co., USA) was used to measure the absorbance of colored substances.

Enzymes assay

Proteolytic activity was evaluated as stated by [24] with some modifications, by measuring the increase in free amino acids split from substrate protein (albumin), during one hour incubation at 30℃. Meanwhile, carboxylesterase activity was determined as stated by [25] using methyln butyrate (MeB) as substrate.

Histological studies

Histological studies were evaluated after 24 h post-exposure to the LC_{50} of the tested larvicides. The LC_{50} of *S. carpocapsa* IJs were 66.93, 38.71, and 25.86 IJs at 48, 72, and 96h PI, respectively. The corresponding LC_{50} values of ZnO NPs were 773.42, 396.70, 273.58, and 182.46ppm at 24, 48, 72, 96h PT, respectively **on survived 4th larval instar midgut.** 10 larvae were sampled for each treatment. Three live larvae were fixed in Bouin for 6 h and then washed with successive concentrations of ethyl alcohol (70, 90, and 100%) to eliminate extra Bouin. After that, larvae were placed in paraffin wax. Paraffin blocks were sectioned with a rotary microtome at a diameter of 5µm and received on glass slides. Sections were deparaffinized, rehydrated, and stained with hematoxylin for 10 min and eosin yellow for 5 min. Sections were dehydrated, cleared in xylene, and then mounted in neutral balsam. Also, untreated larvae were sectioned as previously. All sections were examined using light microscopy and photographed with a digital camera at 40X magnification **[26]**.

Statistical analysis

The mortality data were determined by using analysis through SPSS software, V23, to calculate lethal concentration (LC) and lethal time (LT) values. The mortality percentages were compared through the One-Way Analysis of Variance, followed by Means; the data with the same letters are not significantly different at $P > 0.05$ **[27]**. The biochemical assay was determined as (mean ± SE) and One-Way Analysis of Variance. The data were statistically analyzed by ANOVA using SPSS V. 23.

3. Results

3.1. Characterization of ZnO nanoparticles:

TEM showed nearly mixing of spherical particles with an average size of 28.21- 91.28nm, with good size distribution without aggregation **(Fig. 1).**

3.2. Susceptibility test of A. ipsilon

The results showed that *S. carpocapsa* (nematode IJs) and zinc oxide nanoparticles as larvicides exhibited a high toxic effect against *A. ipsilon* larvae.

S. Carpocapsa had no effect on the tested larvae after 24 hours post-infection. Their lethal effect started after 48 hours of treatment. It exhibited an ascending time and number of IJs in a dose-dependent manner, with maximum mortality at 96h PI (53.33, 66.67, and 86.67% for 20, 40, and 80 IJs / larvae, respectively) **(Fig. 2).**

The insecticidal efficacy represented by the median lethal concentraction (LC_{50}) values of *S. carpocapsa* IJs infection and ZnO NPs treatment. The LC_{50} of *S. carpocapsa* IJs were 66.93, 38.71, and 25.86 IJs at 48, 72, 96h PI, respectively. The corresponding LC_{50} values of ZnO NPs were 773.42, 396.70, 273.58, and 182.46ppm at 24, 48, 72, 96h PT, respectively **(Table 1)**.

According to the speed of killing larvae, the LT_{50} values of *S. carpocapsa* IJs were 153.48, 57.27, and 39.12 h with 20, 40, and 80 IJs/ larvae, respectively. On other hand the LT_{50} values of ZnO NPs were 272.63, 45.62, and 24.77 h with 150, 300, and 450ppm, respectively **(Table 2)**.

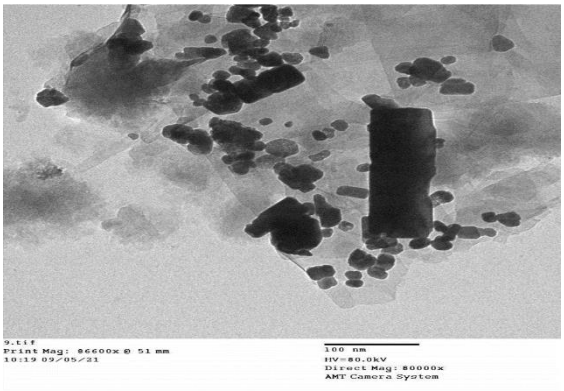

Fig. 1. Transmission electron microscopy (TEM) image of zinc oxide nanoparticles Susceptibility test of *A. ipsilon*

3.3. Biochemical assay

Carboxylesterase activity

Results in **Fig. 5** showed that carboxylesterase levels were significantly decreased ($P< 0.05$) after 24h of treatment with LC_{50} of *S. carpocapsae* as compared to the control group, 514.67, and 6226.00 (ug Meb/min/g.b.wt), respectively. In contrast, after 24h of treatment with LC_{50} of ZnO NPs, carboxylesterase levels were significantly increased ($P< 0.05$) as compared to the control group, 892.00, and 622.0000 (ug Meb/min/g.b.wt), respectively.

Data in **Fig. (3)** showed that the *A. ipsilon* larvae had high susceptibility to ZnO NPs after 24 hours post-treatment (PT), and mortality increased as time passed. The highest mortality percentages after 96h were 53.33, 66.67, and 86.67% for 150, 300, and 450ppm, respectively.

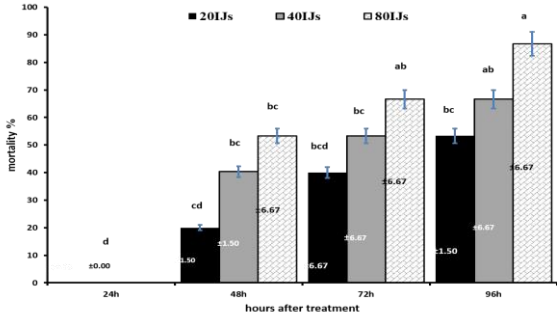

Fig. 2. Mortality percentage of *A. ipsilon* larvae after infection with *S. carpocapsa.*

* Numbers on bars represents the mean plus standard error.

* Different letters indicated to significant differences at ($P \leq 0.05$).

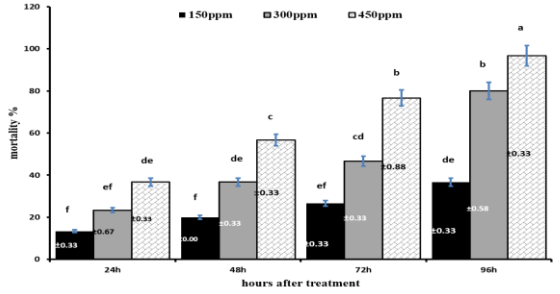

Fig. 3. Mortality percentage of *A. ipsilon* larvae after treatment with zinc oxide nanoparticle.

* Numbers on bars represent the mean plus standard error.

* Different letters indicated significant differences at ($P \leq 0.05$).

Table 1. Insecticidal activity of *S. carpocapsa* IJs infection and zinc oxide nanoparticles against *A. ipsilon* larvae

Treatment \ Lethal concentration			LC_{50}* (Lower-Upper) [a]	LC_{90}* (Lower- Upper) [a]	X^2	Equation
***S. carpocapsa* IJs infection**	**Time post infection**	**24h**	-	-	-	-
		48h	66.93 (50.84- 101.89)	463.51 (347.74- 769.28)	0.22	y= 2.80+1.54*x
		72h	38.71 (20.75- 58.65)	300.05 (235.34- 510.57)	0.04	y= 2.26+ 1.43*x
		96h	25.86 (7.36-38.85)	95.07 (56.50- 1745.39)	0.00	y= 7.94+ 10.94*x
ZnO NP	**Time post treatment**	**24h**	773.42 (440.10- 986.00)	4857.47 (1196.37- 109752.12)	0.11	y= 4.57+ 1.58*x
		48h	396.70 (293.84- 948.89)	1629.10 (774.74- 73496.28)	0.22	y= 5.37+2.07*x
		72h	273.58 (207.85- 358.65)	814.84 (535.34- 2844.57)	1.14	y= 6.62+ 2.72*x
		96h	182.46 (141.23- 216.42)	361.89 (297.28- 520.24)	0.23	y= 7.94+ 10.94*x

*** Concentration of *S. carpocapsa* IJs infection (IJs/ larvae) and concentration of ZnO NP (ppm)**

Table 2. Lethal time values of *S. carpocapsa* IJs infection and zinc oxide nanoparticles against *A. ipsilon* larvae

Treatment \ Lethal time	LT_{50} (h)	LT_{90} (h)

***S. carpocapsa* IJs infection**	20 20 IJs/ larvae	153.48 (90.51-197.21)	1370.99 (871.10- 1592.57)
	40 IJs/ larvae	57.27 (45.59- 83.35)	254.99 (210.75- 321.51)
	80 IJs/ larvae	39.12 (23.75- 50.30)	111.52 (55.92- 176.36)
ZnO NP	150 ppm	272.63 (200.00-387.21)	7907.72 (6871.10- 8092.57)
	300 ppm	45.62 (30.59- 57.35)	200.36 (100.95- 301.44)
	450 ppm	24.77 (15.75- 38.30)	91.54 (75.92- 139.69)

Proteolytic activity

Results in **Fig. 4** revealed that the protease levels significantly decreased (P<0.05) after 24h post-treatment with the LC_{50} of *S. carpocapsae* and ZnO NPs, as compared to the control group: 64.8, 28.2, and 79.6 (µg alanine/min/g.b.wt), respectively.

3.4. Histological effects on midgut

Fig. (6) displayed the histological characteristics of the midgut of survived of *A. ipsilon* 4th larval instar treated with the LC_{50} of the examined substances. **Fig. (6A, B)** of untreated larvae showed that the midgut is normally a simple duct consisting of columnar cells resting on a basement membrane with central nucleus. Columnar cells are scattered apically with goblet cells and basally with regenerative cells. The brush border and the peritrophic membrane are also observed.

Results in **fig. (6D)** showed that after 24h of treatment with the LC_{50} of *S. carpocapsae*, the regenerative cells lost their integrity between the columnar cells and were detached from the basement membrane but retained their distinct integration. The peritrophic membrane was unattached and disintegrated. On the other hand, the gut lumen completely disappeared. On the contrary, after 24h of treatment with LC_{50} of ZnO NPs the midgut showed complete deformity (**fig. 6C**).

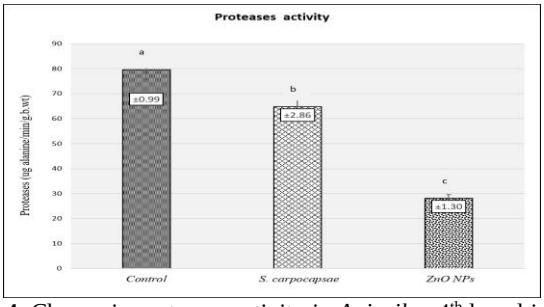

Fig. 4. Change in proteases activity in *A. ipsilon* 4th larval instar after 24h of treatment with LC_{50} of tested larvicides.

* Numbers on bars represent the mean plus standard error.

* Different letters indicated significant differences at ($P \leq 0.05$).

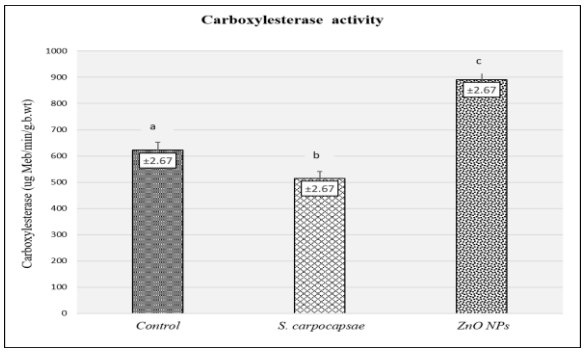

Fig. 5. Change in carboxylesterase activity in *A. ipsilon* 4th larval instar after 24h of treatment with LC_{50} of tested larvicides.

* Numbers on bars represent the mean plus standard error.

* Different letters indicated significant differences at ($P \leq 0.05$).

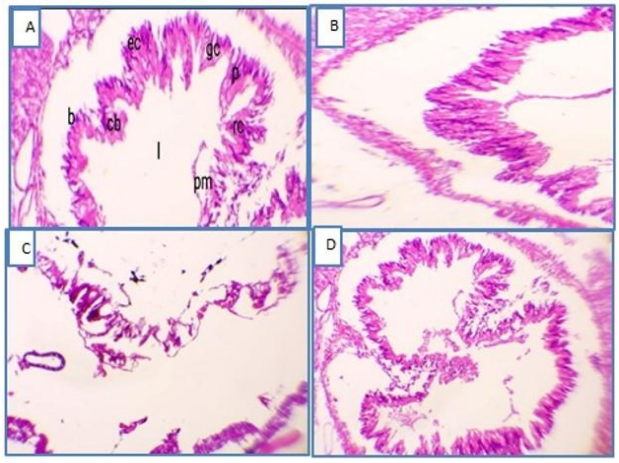

Fig. 6. Photomicrograph of midgut in *A. ipsilon* 4th larval instar after 24h of treatment with LC_{50} of the tested larvicides

A. Control group shows normal midgut structure

B. Magnification of (A)
b: basement membrane, ec: epithelium cells, rc: regenerative cell, gc: goblet cell, p: papillary crypt, cb: ciliated border, pm: peritrophic membrane, l: lumen

C. Treated larvae with ZnO NPs showing complete degeneration in the midgut

D. Treated larvae with *S. carpocapsae* showing a waist in the middle of the midgut

Unequal magnifications of the picture to provide a clearer picture of the shape of the cells after treatment.

4. Discussion

The black cutworm, *A. ipsilon* is considered one of the greatest damaging and subversive lepidopterous pests attacking cotton plants and other vegetable field crops in Egypt. The prospect of replacing the organic insecticides with environmentally safe insecticides to control *A. ipsilon* may conserve the natural enemies in the agro- ecosystem. This study aimed to find an effective method for controlling *A. ipsilon* larvae. The current study evaluated the insecticidal effect of entomopathogenic nematode *S. carpocapsa* and ZnO NPs against the 4th larval instar. Results revealed that *A. ipsilon* larvae exhibited high sensitivity to *S. carpocapsa*. The LC_{50} values of *S. carpocapsa* IJs were 66.93, 38.71, and 25.86ppm at 48, 72, 96 h PT, respectively. The LT_{50} values of *S. carpocapsa* IJs were 153.48, 57.27, and 39.12 h with 20, 40, and 80 IJs/ larvae, respectively. Our results are in harmony with previous studies, which recorded that *S. carpocapsa* caused mortality percentages above 92% in *A. ipsilon* larvae [28-31]. The 3rd and 4th instars recorded high percent mortality, 97% and 83 %, respectively, for S. carpocapsae, while the 2nd and 6th instars had less susceptibility [32, 9]. *S. carpocapsae* efficacy *against A. ipsilon enhanced by* syringing (i.e., light irrigation applied twice daily through warm weather) on average 8% and improved percent mortality (mean 78%, range 75–87%) at 109 nematodes per hectare [33].

A. ipsilon larvae have high susceptibility to ZnO NPs with LC_{50} values 773.42, 396.70, 273.58, and 182.46ppm at 24, 48, 72, 96 h PT, respectively. The LT_{50} values of ZnO NP were 272.63, 45.62, and 24.77h with 150, 300, and 450ppm, respectively. In agreement with these findings, ZnO NPs have been confirmed to be a promising source of a safe method strategy in controlling *Spodoptera frugiperda* and many insect pests with high percent mortality at low doses [34, 15]. Also, these observations corroborate previous studies with different insects such as *Trialeurodes vaporariorum*, the Greenhouse; *Callosobruchus maculatus*, the cowpea beetle; *Sitophilus oryzae*, the whitefly rice weevil and *Tribolium castaneum*, the red flour beetle [35, 36]. The ZnO NPs improved the percent mortality of the 4th larval instar *Spodoptera litura* with an average 27%, in addition to causing malformation in pupae and adults, late emergence, and decreased fecundity and fertility [37].

Biochemical and histological studies were done to gain a satisfactory explanation for the effect of the tested larvicides on *A. ipsilon*. Biochemical results showed that the protease level significantly decreased after treatment with the LC_{50} of *S. carpocapsae* and ZnO NPs after 24h of exposure as compared to the control group. In agreement with our results, the level of protease enzyme in *A. ipsilon* 4th larval instar decreased after 16h of infection with *Heterorhabditis zealandicaand* and *Steinernema abbasi* compared to the control. Protease enzyme activity decreased due to the digestion of the proteins in the insect body by the symbiotic bacteria released by entomopathogenic nematodes after 16h PT [38]. Other studies have also shown that total protein levels significantly decreased after treatment with normal or irradiated *Heterorhabditis bacteriophora* or *Steinernema scapterisci* compared to the control [39].

Regarding our findings about the effect of ZnO NPs on the protease enzyme, similar results showed that ZnO NPs decreased the mid-gut protease, α-amylase, activity in *Callosobruchus maculatus*, the pulse beetle [40]. Carboxylesterase (CarE) is the primary hydrolytic and metabolic enzyme that breaks ester bonds [41]. The detoxifying enzymes play a critical role in pesticide resistance and are associated with resistance in many insects to many insecticide classes [42, 43]. According to the role of metabolic enzymes, carboxylesterase activity is used as a biochemical indicator for insecticide resistance in many pests. Results showed that carboxylesterase level significantly decreased after treatment with LC_{50} of *S. carpocapsae* after 24h from exposure compared to the control group. On the other hand, carboxylesterase levels were significantly increased (P<0.05) after treatment with LC_{50} of ZnO NPs as compared to the control group. One of the studies that evaluated the effect of pathogenic nematode on *A. ipsilon* found that the level carboxylesterase was increased in *A. ipsilon* 4th larval instar, decreased after 16 h of infection with *Heterorhabditis zealandicaand* and *Steinernema abbasi* as compared to the control [38].

Histological results showed that the *S. carpocapsa* and ZnO NPs exhibited histological impacts against the midgut of the surviving *A. ipsilon* 4th larval instar after treatment. Damage to the midgut, digestive issues, and nutritional metabolism abnormalities are observed as physiological changes, as proven by a biochemical test (proteolytic activity). In many non-nerve tissues, tested formulations could cause oxidative stress and apoptosis [44, 45].

Our results are in harmony with previous studies, which recorded that the Tca toxins, the mcf gene product, and a 17-kDa pilin are gram-negative bacteria that live in symbiosis with entomopathogenic nematodes belonging to the genera *Steinernema* and *Heterorhabditis* caused damage to midgut as well as destroyed the epithelial cells of *Galleria mellonella* larvae [46]. Also, Zinc sulfate caused elongation and separation in midgut epithelial cells, rupture in the peritrophic membrane, and degeneration of the surrounding muscles in the red palm weevil, *Rhynchophorus ferrugineus* larvae [47].

5. Conclusion

Based on the results of this study, it is possible to confirm that the two tested control strategies (*Steinernema carpocapsae* and zinc oxide nanoparticles) are highly effective against *Agrotis ipsilon* larvae, achieving a high mortality rate. Therefore, it can be concluded that the use of chemical insecticides should be minimized, and *S. carpocapsae* and ZnO NPs can be used as alternatives for controlling *A. ipsilon*.

Conflicts

The author has no conflicts of interest that are concerned with this article.

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