The Humoral Immunological Response and Bio-Efficiency Evaluation to Entomopathogenic Nematodes as A Biopesticide on *Spodoptera littoralis* (Boisd)

Rima Shahin,[1*] Wael M. Khamis [2] and Ahmed M. El-Sabrout[1]

ABSTRACT

The current study aims to investigate the residual toxicity and nutritional indices of entomopathogenic nematodes (EPNs), as well as immunological response to an infection in *Spodoptera littoralis*. Compared to the individual impacts of *Steinernema carpocapsae, Heterorhabditis indica* and EPNs binary combinations showed a higher pathogenicity to *S. littoralis* according to mortality, physiological responses, and humoral immune. The anti-feedant activity percentages of *H. indica* and *S. carpocapsae* treated larvae had a more potent effect than their binary mixture at LC_{35}, compared to full-feeding activity in the control. The biochemical characteristics of infected hemolymph showed a significant variation, not only in the total protein concentration, but also in the appearance of the new protein bands profile. An additional band, with a molecular weight of ~79 KDa was observed at immunized hemolymph, it has been postulated that it may be the phenoloxidase (PO), which is in close agreement with the significant induction of PO activity after infection. Interestingly, the immunised hemolymph of the larvae infected with binary combinations of EPNs showed a notably increased intensity of the major proteins and a rise in the number of antimicrobial peptides with molecular weights under 30 KDa. However, the significant reduction of total protein count and PO level, which ultimately resulting in larval death, is evidence that the binary mixture of the investigated nematodes is highly suppressed *S. Littoralis* larval immune system.

Keywords: entomopathogenic nematodes, humoral immunological response, bio-efficiency evaluation, *Spodoptera littoralis* (Boisd).

INTRODUCTION

Spodoptera littoralis (Boisd.) is the most highly destructive polyphagous insect that consumes plants from 44 different families, including 87 species, within tropical and subtropical range including grasses, crucifers, legumes and fruit trees. Egypt is where it first appeared, and today it can be found in Africa, the Canary Islands, the Middle East, and portions of Mediterranean Europe, such as Madeira and the Balearic Islands (Salama and Shoukry, 1972). It is highly destructive and infest the main economic plants in different areas such as, cotton in Egypt, vegetables in Africa, plant and flower cultivation in greenhouses (Hosny, *et al*., 1986). *S. Littoralis* is primarily controlled by synthetic chemical insecticides, but due to the potential for ecological disruption, the emergence of resistance, and the overuse of these products, which raises concerns about environmental contamination and the extinction of natural enemies, has led to find better options. Nowadays, biological control approaches are now required to control *S. littoralis*, including the use of entomopathogenic agents, transgenic plants, insect growth regulators, host plant resistance and slow-release pheromone formulations for mating disruption (Rashwan *et al*., 1991).

Entomopathogenic Nematodes (EPNs), a type of biopesticide that has good target specificity and is thought to be environmentally harmless, are now being recognized as significant alternatives to chemical insecticides (Burnell and Stock, 2000). EPNs are categorised into 23 families, the most common is *Steinernematidae* and *Heterorhabditidae* which well known for their soil-dwelling organisms that penetrate the insect cuticle and release symbiotic bacteria into the insect's gut and hemocoel (Shamseldean *et al.,* 2013; Vashisth *et al.,* 2013). The symbiotic bacteria secrete protoxins, proteases and other insecticidal compounds, which inhibit the host's immune system and resulting in deadly septicemia within 48-72 hrs (Toubarroa *et al.,* 2009; Ullah *et al*., 2014; Jung and Kim, 2007). The most significant and successful insects are found in the order of Lepidoptera, and it is thought that their successful immune systems are the reason for this. The cascade of immunological processes starts as soon as disease agents and parasites enter the hemocel, resulting in simultaneous humoral and cellular defence reactions (Gillespie *et al*., 1997; Söderhäll and Cerenius, 1998). Cellular immune responses such as, coagulation, melanotic encapsulation, , nodule formation and phagocytosis (Ling and Yu, 2005; Strand, 2008). Antimicrobial peptides (AMP) and phenoloxidase (PO) both play crucial roles in humoral defence, which in turn triggers humoral immunity (Lavine and Strand, 2002; Lemaitre and Hoffmann, 2007). Insects synthesise AMPs, which typically have a wide range of

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[1] Department of Applied Entomology and Zoology, Faculty of Agriculture (El-Shatby), Alexandria University, Alexandria 21545, Egypt
[2]Plant Protection Research Institute, Agriculture Research Center, Al-Sabhia, Alexandria, Egypt
Corresponding Author: ✉ Rima Shahin
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antibacterial, antiviral, and antifungal properties (Patocka *et al*., 201٩). The antibacterial activity is directly correlated with the ability of AMPs to interact with the surface of the cell membrane and change the permeability of the membrane (Ongey *et al*., 2018). AMPs when move into the pathogen, they can potentially cause destruct the components of the cytoplasm such as DNA and enzymes (Silvestro and Axelsen, 2000). The prophenoloxidase (PPO) cascade After activation, not only trigger both cellular and humoral immunity but also cause melanization around invasive infections (Lemaitre and Hoffmann, 2007). During the melanization process, intermediate compounds that created have strong antibacterial properties (Zhao et al., 2007). Studying the immunological response and defensive strategies of *S. littoralis* against the intruder EPNs would help us better understand their defence mechanism. This study focused on evaluating the bio-efficiency of EPNs as biopesticide of *S. littorals* and quantitative and qualitative analysis of AMPs, other immune proteins, as well as the activation level of PO enzyme, to identify the EPNs' mode of action within the insect's body and get rid of it.

MATERIAL AND METHODS

Insect rearing:

Samples of egg-masses of *S. Littoralis* were collected from various infested crops in different regions of Al-Behaira, Egypt. The egg-masses were transferred to laboratory for rearing under certain conditions (27 ± 2 °C, RH 60 ± 5 %) according to El-Defrawi *et al.* (1964) into a purposed-built incubator equipped with digital thermostat, hygrometer and 24 hrs-automatic shut-off timer. *S. littoralis*, larvae were fed on fresh castor leaves, *Ricinus communis* (L.). A susceptible laboratory strain (LS) of *S. Littoralis* was obtained after almost 7 generations. Then, the 4th instar larvae were nominated to the evaluation of toxicity, nutritional indices and gel-electrophoresis experiments as well as semi-field trials under the same foregoing conditions in laboratory.

Entomopathogenic nematodes cultures:

Water suspension samples of *Steinernema carpocapsae* and *Heterorhabditis indica* isolates was obtained from department of Pest Physiology, Plant Protection Research Institute, Agriculture Research Center, Cairo. It was identified according to their morphological characters (Doliniski *et al*., 2008; Ganguly and Singh, 2000). Survival infective juveniles (IJs) ≤ 1week after inoculum were used in laboratory and field experiments (Kalia *et al*., 2014). Samples of isolated EPNs were stored in portable cool boxes within few hours when transferred to the laboratory of integrated protection, Alexandria or even to the location of field trials. The isolated EPNs were placed in polyurethane foams in darkness at 25 ± 2 ºC few days prior to laboratory or semi-field experiments (Ramakuwela *et al*., 2015; Paschapur *et al*., 2017).

EPNs bioassay:

Stock solutions of the isolated EPNs were counted for their initial inoculum of IJs ml^{-1} using stereoscopic microscope (10X). The second day of 4th instar larvae of *S. littoralis* were subjected individually to concentrations of 5, 10, 20, 40, 80 and 150 of IJs ml^{-1} in sterile plates (3.5 cm diameter x 2.5 cm depth) lined with moistened paper tissue. The paper tissue technic was used to evaluate the dose response of *S. littoralis* to the endo-parasitic activity of the tested EPNs. Control treatment installed only with sterile water. Each concentration contained set of 10 separated plates. Each plate received one larva. Each treatment was repeated three times. Therefore, each EPN treatment comprised 180 larvae severally. Incubation condition was adjusted at 25 ± 2 °C, RH 60 ± 5 %. Mortality percentages were recorded at 48 hrs, corrected by formula of Abbott (1925) and submitted to probit analysis performed by Finney (1971). The sub-lethal concentrations were expressed by number of IJs ml^{-1} for each larva.

Assay of nutritional indices:

A bioassay of castor oil leaf disc (diameter, 3 cm) contaminated with the tested EPNs was used to check the nutritional indices of the second day of 4th instar larvae of *S. littoralis* at 48 hrs of post infection period (PIP). Nutritional parameters mainly included anti-feedant activity, relative consumption rate (RCR), relative growth rate (RGR), efficient of conversion of ingested food (ECI), efficiency of conversion of digested food (ECD), approximate digestibility (AD) and consumption Index (CI). Using micropipette applicator to spread LC_{35} values for each of *S. carpocapsae*, *H. indica* and their binary mixture overall the surface area of leaf discs in compare to control treatment (treated only with distilled water). The contaminated leaf discs were left for (~ 10 min.) to dry before being introduced to the larvae. One treated leaf disc lined the bottom of each well plate. Thereafter, one (24 hrs pre-starved) larvae was introduced in each well plate and allowed to feed for 48 hrs. Each treatment was replicated three times. Each replicate comprised of a group of ten severally larvae. Therefore, each treatment comprised of totally 30 individual larvae. Extinct and consumed leaf discs were replaced by new every 24 hrs whenever needed. Along the 48 hrs of PIP, means of weights of survival larvae were converted from fresh to dry weight ratios by using an oven at 50 ºC for 24 hrs (*Dermott* and *Paterson, 1974).* The initial fresh weights of treated leaves were converted to dry weight ratios by

using blank replicates (without larvae) to calculate the moisture content loss during the 48 hrs of PIP (Candy and Baker, 2002). The anti-feedant activity percentages were calculated by weighting the leaf disc according to Saleh *et al*. (1986) as well as other nutritional indices parameters were calculated by the formulas of Waldbauer (1968).

$$\text{Anti-feedant activity percentage} = \left(1 - \frac{\text{percentage of treated leaf eaten}}{\text{percentage of untreated leaf eaten}}\right) \times 100$$

Nutritional indices formula:

Relative consumption rate (RCR) = E / T A.

Relative growth rate (RGR) = P / T A.

Efficiency of conversion of ingested food (ECI) = (P x 100) / E.

Efficiency of conversion of digested food (ECD) = P / (E - F).

Approximate digestibility (AD) = (E - F) / E.

Consumption Index (CI) = E / A.

Where:

E = dry weight of food eaten; T = duration of experimental period; A = mean of dry weight of larvae during T; P = dry weight gain of insect; F = dry weight feces of larvae during T.

Co-toxicity factor of binary mixtures of the selected entomopathogenic nematodes:

The 4th larval instars of *S. littoralis* were subjected severally in well sterile plates, under the same forgoing condition of toxicity test, to binary mixtures of the tested EPNs with alternative ratios between their values of LC_{35} and LC_{50} versus the control treatment at 48 hrs post treatment. Each binary mixture was repeated 3 times. Each replicate contained 10 larvae. The joint action of each binary mixture was expressed by using the co-toxicity factor's (CTF) formula performed by Mansour *et al*. (1966).

$$CTF = \left(\frac{\text{observed mortality percentage - expected mortality percentage}}{\text{expected mortality percentage}}\right) \times 100$$

Where CTF indicates the following actions:

Potentiation (≥ +20); antagonism (< - 20); additive effect (from - 20 up to + 20).

Observed mortality percentage = actual mortality percentage of the binary mixture of EPNs.

Expected mortality percentage = sum of mortality percentages of each EPN treatment alone that used in binary mixture.

Field trials:

The semi-field experiments were accomplished in Al-Qnawiyah village, El-Behira governorate in early June of each growing seasons of cotton crop (variety, Giza 86) in 2021 and 2022. Cotton crop plantations disciplined the guidelines of crop management practices (Gibbs *et al*., 2005; Directorate Plant Production, 2016). Each treatment was assigned to three replicated plots (30 $m^{2)}$. The field trials were submitted to a randomized complete block design. Foliar sprays of all treatments were conducted by hand compression spray applicator (10 L capacity). The selected EPN treatments alone (at rate ≡ LC_{90} values) and their binary mixture (at rate ≡ LC_{50} values) were applied in a sufficient minimum total spray volume (1 L) for covering the vegetative part of the plants in each plot (Hofman and Solseng, 2018). Thus, the selected EPN treatments were applied alone with rates equivalent to their LC_{90} values in 1 L^{-1} $plot^{-1}$. The binary mixture of the tested EPNs with rate ≡ their LC_{50} values in 1 L^{-1} $plot^{-1}$ was chosen for field trial as it realized the highest additive action in laboratory (Table 1 and 3). Control plots were sprayed only with water. Samples of mid-aged leaves from treated and untreated plots were collected in perforated bags at intervals of 0 (3hrs), 2, 4, 6, 9 and 12 days aftert-treatments (DAT). The samples were transferred to laboratory to resume the susceptibility tests of each treatment throughout 48 hrs on the 4th instars under 25 ± 2 °C, RH 60 ± 5 %. Equally nourishing portions of cotton leaf were introduced in glass cups (200 cm^3) to feed ten (24 hrs pre-starved) 4th instar. Three replicates were used in each treatment. Mortality and residual toxicity were recorded at 48 hrs of exposure for each interval and corrected by formula of Abbott (1925).

b- Biochemical parameters:

Biochemical tests were accomplished at 48 hrs of post infection period (PIP) with EPNs (at LC_{35} values) and their binary mixtures (at LC_{35}, realizing the lowest observed mortality) on the second day-4th instar larvae of *S. littoralis*.

Phenoloxidase (PO) assay:

By monitoring the synthesis of dopachrome from L-dopa substrate, the (PO) activity in hemolymph samples from healthy or infected *S. littoralis* larvae were determined spectrophotometrically after two days of PIP. Eight µl of hemolymph were added to 1mL of L-dopa buffer (0.004M L-dopa -10mM Tris–HCl, pH 7.2) for each of the experiments, as a blank, the L-dopa buffer was utilized. A microplate reader was used to measure the rise in absorbance at 490 nm at 20 °C for 30 minutes at one-minute intervals.

Estimation of total hemolymph protein (THP):

Hemolymph was extracted and placed in sterile tubes with a few phenylthiourea crystals from control and infected *S. littoralis* larvae after 48 hrs of PIP. The samples were then centrifuged at 10,000 g for 10 min to remove hemocytes and cell debris. Protein concentrations in samples of hemolymph were

calculated using Bradford's technique (**Bradford, 1976**). Bovine serum albumin (BSA) dilutions were used to generate a standard curve for protein estimation.

SDS-PAGE of hemolymph:

Using 10% resolving and 4% stacking polyacrylamide gel (SDS-PAGE) (Laemmli, 1970), cell-free hemolymph was applied on a Bio-Rad electrophoresis device with the power supply set at 75 V during the stacking gel then 125 V till the end of the electrophoresis. Hemolymph samples were run alongside a wide-range (10-225 kDa) Protein Marker (Sigma) to estimate and compare changes in protein samples. Ten µg sample from both healthy and infected larvae was placed in each gel lane. Coomasie Brilliant Blue R250 was used to stain the gels. Total lab software was used to analyse the gels (Microsoft version 11.0).

Statistical Analysis:

The obtained data of the tested EPNs concerning feeding indices and co-toxicity in laboratory experiments as well as mortality percentages and residual toxicities in field trials and biochemical studies were subjected to analysis of variance (ANOVA) using statistical software (SAS, 2002) at LSD between treatments (P = 0.05 %).

RESULTS

Toxicity of the selected entomopathogenic nematodes on *S. littoralis*

Toxicity studies were conducted to estimate the values of LC_{35}, LC_{50} and LC_{90} of the selected EPNs on the 4th instars of *S. littoralis* after 48 hrs of post infection period (PIP) under the laboratory condition (Table 1). The lethal concentration of *S. carocapsa* showed higher toxic effects with 9.88, 16.65 and 94.36 IJs ml / larva compared to *H. indica*, which realized 11.2, 20.32 and 150.76 IJs ml^{-1} $larva^{-1}$ for their LC_{35}, LC_{50} and LC_{90}, respectively.

Nutritional indices of the selected entomopathogenic nematodes on *S. littoralis*

Data of nutritional indices of the survival 4th instars of *S. littoralis* treated with LC_{35} values of EPNs after 48 hrs of PIP were represented in table (2). Anti-feedant activity percentages in both treatments of *S. carpocapsae* and *H. indica* were equally prevailed with 8.46 and 7.87 %, respectively. While it was 4.30 % for the binary mixture of EPNs at LC_{35} compared to absence of anti-feedant activity in the control. Approximate digestibility percentage in 4th instars larvae showed significant declines whenever treated with *S. carpocapsae* (138.63 %) and *H. indica* (121.55 %) in compare to its highest equipollent values between binary mixture of the tested EPNs and the control. Consumption Index values had no significant differences between all the treatments except CI of *H. indica* (7.50 %) were evidently lower than the control. Efficiency of conversion of digested food percentage showed highest equipollent values between *S. carpocapsae* (7.81 %) and *H. indica* (9.73 %). However, ECD % of binary mixture of EPNs at LC_{35} (4.22 %) showed a highest significant decrease in compare to all treatments. Relative consumption rate, RGR and ECI of other treatments of EPN were significantly lower than the control. Moreover, no significant differences of the values of CI, RCR, RGR and ECI were found between all the treatments of EPN (Table 2).

Table 1. Toxicity of the tested entomopathogenic nematodes on 4th instar larvae of *Spodoptera littorals* after 48 hrs of exposure

Entomopathogenic nematode	Concentrations	IJs* ml / larva (Fiducial limits)		Slope ± SE**	χ^{2***}	*df*
Steinernema carpocapsae	LC_{35}	9.88	(6.00-16.30)	1.61± 0.29	1.65	4
	LC_{50}	16.65	(11.45-24.21)			
	LC_{90}	94.36	(56.24 – 158.31)			
Heterorhabditis indica	LC_{35}	11.20	(4.93-25.08)			
	LC_{50}	20.32	(11.28- 36.59)	1.26 ± 0.21	4.26	5
	LC_{90}	150.76	(85.64 – 265.40)			

*****Infective juveniles; **SE: Standard error; *** χ^2: Chi-Square

Table 2. Effects of LC_{35} concentrations of the tested entomopathogenic nematodes on the nutritional indices of the survival 4th instar larvae of *Spodoptera littoralis* after 48 hrs of exposure

Treatments	Nutritional indices parameters ± Standard deviation									
	Anti-feedant activity %	AD %[1]	CI[2]	ECD %[3]	RCR[4] (mg/mg/day)	RGR[5] (mg/mg/day)	ECI[6] %	E[7] (mg)	P[8] (mg)	F[9] (mg)
Steinernema carpocapsae	8.46[a] ±0.58	138.63[b] ±1.53	8.35[ba] ±2.72	7.81[bc] ±1.16	3.74[b] ±1.14	0.11[b] ±0.03	5.53[b] ±0.81	203.38[c] ±5.78	6.22[b] ±0.80	51.00[c] ±1.73
Heterorhabditis indica	7.87[a] ±1.80	121.55[c] ±8.20	7.50[b] ±1.35	9.73[ba] ±3.39	3.43[b] ±0.57	0.10[b] ±0.03	5.30[b] ±1.161	236.00[cb] ±34.44	6.89[b] ±1.85	67.33[b] ±2.31
Binary mixture of EPN at LC_{35}	4.30[b] ±0.47	158.50[a] ±0.78	10.08[ba] ±0.84	4.22[c] ±1.22	4.59[b] ±0.24	0.07[b] ±0.03	3.14[b] ±0.91	248.82[b] ±12.31	2.93[b] ±0.79	45.33[d] ±0.58
Control	0.00[c] ±0.00	157.23[a] ±3.32	11.33[a] ±0.50	12.64[a] ±3.43	18.72[a] ±2.32	0.68[a] ±0.36	9.50[a] ±2.20	493.27[a] ±29.05	25.15[a] ±4.78	131.00[a] ±2.00
$L.S.D_{0.05}$	1.83	9.73	3.00	4.81	2.50	0.34	2.61	44.31	4.94	3.35

Approximate digestibility[1]; Consumption Index[2]; Efficiency of conversion of digested food %[3]; Relative consumption rate[4]; Relative growth rate[5]; Efficiency of conversion of ingested food %[6]; Dried weight of eaten food[7]; Dry gained weight of larvae[8]; Faeces[9].

- Means for each column with the same letter are not significantly different according to the $LSD_{0.05}$.

Co-toxicity factor of binary mixtures of the tested entomopathogenic nematodes

All binary mixtures of the tested EPN showed additive effects on the 4th instar larvae of *S. littoralis* (Table 3). Overall the tested binary mixtures, the ratio of LC_{50} : LC_{50} showed highest additive effect with CTF of 16.05. In the second rank, *S. carpocapsae* at LC_{50} + *H. indica* at LC_{35} came with CTF of 10.01. Meanwhile, *S. carpocapsae* + *H. indica* at LC_{35} : LC_{35} and LC_{35} : LC_{50} had the lowest CTFs (Table 3).

Residual toxicity of Semi-field trials

percentages of mortality and residual toxicity of the tested EPNs alone (at rates ≡ LC_{90}) and their equally binary mixture (at rate ≡LC_{50} realized highest additive effect) in compared to the control treatment against the 4th instar larvae of *S. littoralis* after 48 hrs post infection period (PIP) were conducted during the growing seasons of cotton crop in 2021 and 2022 (Table 4 and 5).

a. *Season 2021*

The obtained data of the binary mixture of the tested EPNs (at rate ≡LC_{50}) realized the most potent toxic effect that excelled all the individual treatments of EPN (at rates ≡ LC_{90}) along the 12th DATs. However, high toxic effects attained by the individual treatments (at rates ≡ LC_{90}) of *S. carpocapsae* surpassed *H. indica* at 3, 6 and 9 days after treatment (DAT). Moreover, there were no significant differences between both of *S. carpocapsae* and *H. indica* at 0 and 12 DAT (Table 4). The results of overall mean of mortality percentages of the binary mixture of the tested EPNs (80.00 %) were significantly higher than *S. carpocapsae* (74.00 %) and *H. indica* (64.67 %) (Table 4).

b. *Season 2022*

Data of the 4th instars larvae affirmed equipollent toxic effects for rates ≡ LC_{90} of *S. carpocapsae* and *H. indica* along the 12th DATs. In the same pace, the binary mixture of EPNs (at rate ≡LC_{50}) transcends all the individual treatments of EPN (at rate ≡LC_{90}) along the 12th DATs (Table 5). Eventually, the results of overall mean of mortality percentages showed significant differences between each of the binary mixture of EPNs (74.44 %) and *H. indica* (62.22 %) (Table 5).

Table 3. Co-toxicity factor of binary mixtures of the tested entomopathogenic nematodes on 4th instar larvae of *Spodoptera littoralis* after 48 hrs of exposure

Binary mixtures			Expected mortality %	Observed mortality %	Co-toxicity factor	Action type
S. carpocapsae	**:**	***H. indica***				
LC_{50}	:	LC_{50}	83.33	96.67	16.05	additive
LC_{50}	:	LC_{35}	66.66	73.33	10.01	additive
LC_{35}	:	LC_{35}	53.33	56.67	6.24	additive
LC_{35}	:	LC_{50}	70.00	73.33	4.76	additive

[1]Observed mortality percentage = actual mortality percentage of the binary mixture of EPNs.

[2]Expected mortality percentage = sum of mortality percentages of each EPN treatment alone that used in binary mixture.

Table 4. Mortality percentages of the tested entomopathogenic nematodes against the 4th instar larvae of *Spodoptera littoralis* in semi-field trials after 48 hrs post infection period (PIP), season 2021

Treatments	Mean of mortality % ± SD* after 48 hrs of exposure along consecutive days after treatments					Overall mean of mortality%
	0	3	6	9	12	
Steinernema carpocapsae at LC_{90}	86.67^{ab} ±5.77	86.67^{a} ±5.77	80.00^{a} ±0.00	70.00^{a} ±0.00	46.67^{b} ±5.77	74.00^{b} ±3.33
Heterorhabditis indica at LC_{90}	76.67^{b} ±11.55	76.67^{b} ±5.77	66.67^{b} ±5.77	56.67^{b} ±5.77	46.67^{b} ±5.77	64.67^{c} ±3.33
EPN mixture at LC_{50}	90.00^{a} ±0.00	93.33^{a} ±5.77	83.33^{a} ±5.77	76.67a ±5.77	56.67^{a} ±5.77	80.00^{a} ±3.33
Control	0.00^{c} ±0.00	0.00^{c} ±0.00	0.00^{c} ±0.00	0.00^{c} ±0.00	0.00^{c} ±0.00	0.00^{d} ±0.00
L.S.D$_{0.05}$	12.15	9.42	7.69	7.69	9.41	4.83

*Standard deviation.

- Mortality percentages in each column separately (representing each interval of exposure vs. all treatments) with the same letter are not significantly different according to the $LSD_{0.05}$.
- Means of overall mortality percentages of the treatments with the same letter are not significantly different according to the $LSD_{0.05}$.

Table 5. Mortality percentages of the tested entomopathogenic nematodes against the 4th instar larvae of *Spodoptera littoralis* in semi-field trials after 48 hrs of exposure, season 2022

Treatments	Mean of mortality % ± SD* after 48 hrs of exposure along consecutive days after treatments					Overall mean of mortality %
	0	3	6	9	12	
Steinernema carpocapsae at LC_{90}	83.33^{ba} ±5.77	80.00^{ba} ±10.00	76.67^{ba} ±5.77	63.33^{ba} ±5.77	43.33^{ba} ±5.77	69.33^{a} ±1.92
Heterorhabditis indica at LC_{90}	76.67^{b} ±5.77	70.00^{b} ±0.00	70.00^{b} ±0.00	53.33^{b} ±5.77	36.67^{b} ±5.77	61.33^{b} ±3.85
EPN mixture at LC_{50}	86.67^{a} ±5.77	86.67^{a} ±5.77	83.33^{a} ±5.77	70.00^{a} ±10.00	50.00^{a} ±10.00	75.33^{a} ±5.09
Control	0.00^{c} ±0.00	0.00^{c} ±0.00	0.00^{c} ±0.00	0.00^{c} ±0.00	0.00^{c} ±0.00	0.00^{c} ±0.00
L.S.D$_{0.05}$	9.41	10.87	7.69	12.15	12.15	6.28

*Standard deviation.

- Mortality percentages in each column separately (representing each interval of exposure vs. all treatments) with the same letter are not significantly different according to the $LSD_{0.05}$.
- Means of overall mortality percentages of the treatments with the same letter are not significantly different according to the $LSD_{0.05}$.

Phenoloxidase (PO) activity:

Changes in PO enzyme activity between treated and control larvae of 4th instar were determined 48 hrs after nematodes infection (Fig. 1). The result showed that there was a noticeable difference of PO enzyme activity between the control (0.51 U/µl/min) and infected larvae. The hemolymph of infected larvae with an EPNs binary mixture had the lowest PO activity (0.61 U/µl/min), which was then followed by a noticeable induction during the treatment with *S. carpocapsae* and *H. indica*. However, there were considerable variations in PO activity at *S. carpocapsae* (0.78 U/µl/min), and an increase in parasite larval activity at *H. indica* (0.82 U/µl/min).

Effect of Entomopathogenic nematodes on the total protein in hemolymph (THP):

The total protein in hemolymph of infected and non-infected larvae groups is displayed in (Fig. 2). The mean values obtained from controls were 3.98 mg/ml on the fourth instar larvae, which was substantially higher than the parasitized groups. However, in parasitized larvae, a marked decrease in protein content (1.42 mg/ml) was found in the binary mixture of the examined EPNs over the individual infection. The mean protein concentration of *H. indica*, treated larvae, was higher than that of *S. carpocapsae* (2.92 mg/ml 2.17 mg/ml), respectively.

Electrophoretic patterns of infected and non-infected larvae hemolymph proteins:

SDS-PAGE was used to analyse the hemolymph protein profile of *S. littoralis* fourth instar larvae, loading the lane with alternate fractions of control and infected hemplymph (Fig. 3). The electrophoretic profile identified eight major bands of proteins in the hemolymph with molecular weights of ~ 72, ~62, ~48, ~34, ~30, ~19, ~17 and ~11 kDa; their molecular weights ranged from ~4 to ~300 kDa. In control and infected larvae, the ~ 72, ~34, and ~17 kDa were more defined. The infectious larvae of *S. carocapsa* and *H. indica* in particular showed a novel, intense band that migrated at an apparent molecular weight of ~79, which was higher than binary combinations of the tested EPNs. In general, the hemolymph protein bands of the infected *S. carocapsa* and *H. indica* larvae showed a considerable reduction in intensity, and a consistent increase in the ~17 kDa over control was identified. When the larvae were infected with binary combinations of the evaluated EPNs, there was a noticeably increased intensity of the major proteins and an increase in the number of hemolymph protein bands, particularly proteins with molecular weights under ~30 KDa.

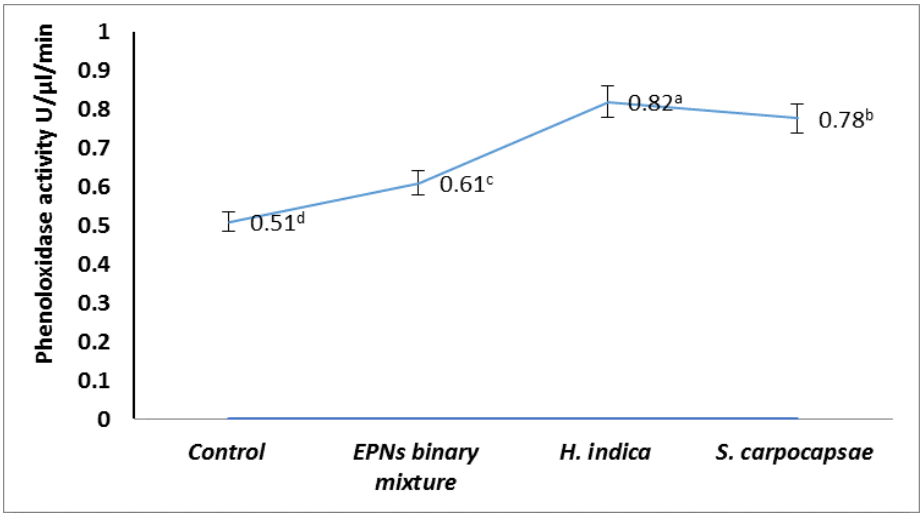

Figure 1. Phenoloxidase activity (U/µl/min) in hemolymph of infected and non-infected larvae with entomopathogenic nematodes, means with the same letter are not significantly different according to the LSD0.05= 0.025

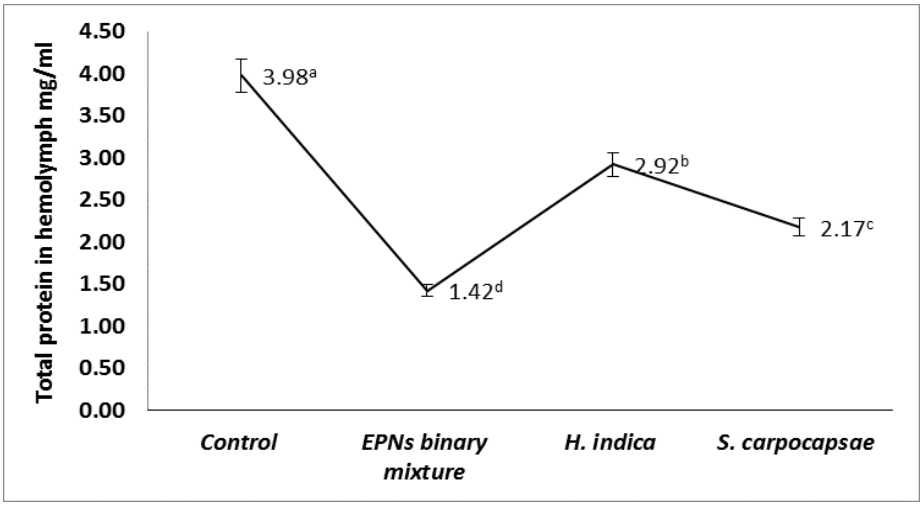

Figure 2. Total hemolymph protein content in infected and non-infected larvae of *S. littoralis*, means with the same letter are not significantly different according to the $LSD_{0.05}$= 0.028.

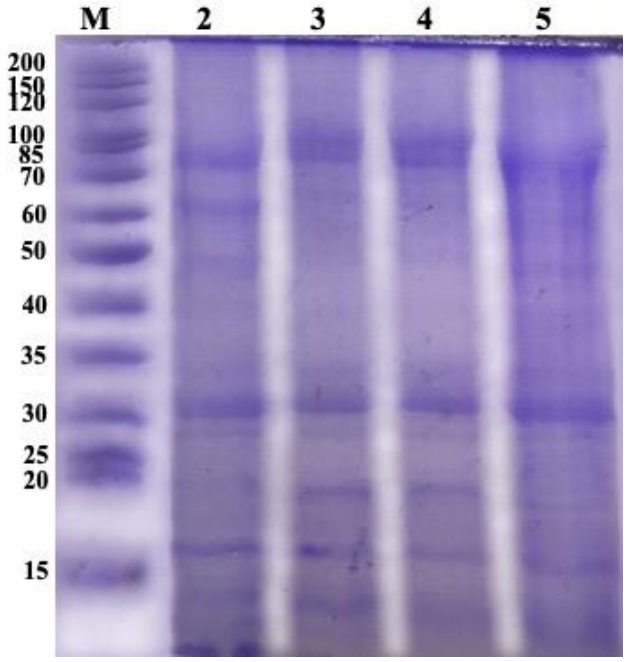

Figure 3. Electrophoretic protein profile 10% SDS-PAGE of *S. littoralis* hemolymph, lane 1: (M) standard protein marker, lane 2: (C) control, lane 3: *H. indica*, lane 4: *S. carocapsa* and lane 5: EPNs combinations. Each lane received samples containing 10 µg of total protein

DISCUSSION

As far as we know, EPNs isolates are one of the novel biopesticide agents that could significantly alter and rival the conventional insecticides particularly in target specificity on insects and adverse effect on environment (Bhat *et al*., 2021). Lepidopterans are recognized to possess successful immune systems that trigger the humoral and cellular defence reactions (Dillman *et al*., 2012). In order to measure how *S. littoralis* larvae could respond to the tested EPNs and their binary mixtures, our study leaned to evaluate dose response assay, molecular protein marker and residual efficacy.

Firstly, we demonstrated that the toxicity values of LC_{35}, LC_{50} and LC_{90} of *S. carocapsa* possessed high toxicities more than *H. indica* against the 4th instar larvae of *S. littoralis*. Based on CTF, all binary mixtures of the tested EPNs exhibited additive effects specially at the ratio of LC_{50} : LC_{50} against the 4th instars in compare to their tested EPNs alone. According to numerous studies conducted on dose response assays, sub-lethal doses of *S. carocapsa* isolates were observed to own higher toxicity than *H. indica* against the latest instars of lepidopterans for instance, *Spodoptera litura*, *S. littoralis* and *Helicoverpa armigera* at different times of exposure (Holajjer *et al*., 2014; Prasad *et al*., 2012; Gomaa *et al*., 2020). In addition, the obtained data of feeding indices on the 4th instars treated with the EPNs and their binary mixture at LC_{35} after 48 hrs post infection period (PIP) showed that anti-feedant activity percentages in *S. carpocapsae* and *H. indica* had equipollent potent effect more than their binary mixture at LC_{35}, while full-feeding activity was shown in the control. In addition, AD had significant decreases for both individual EPN treatments versus to their binary mixture and control. Moreover, RCR, RGR and ECI had equipollent significant declines overall the tested EPNs in compared to the control. Furthermore, ECD reached its lowest values in binary mixture of the tested EPNs and increased gradually in both individual EPN treatments and reached its maximum value in control treatment. In this study, all the impairment aspects of nutritional indices and growth rate in the treated larvae at the 48 hrs post treatment with the selected EPNs and their mixture may be attributed to the ability of EPNs to travel across the intestinal barrier of the larvae's tract within 8 hrs post infestation and followed by releasing their symbiotic bacteria that could multiply at 15 hrs post infestation to cause septicemia in the hemocoel of the caterpillar (Sicard *et al*., 2004; Lalitha *et al*., 2018; Huot *et al*., 2019). Thence, 24 hrs may be a sufficient time for EPN's penetration and completion of bacterial multiplications in insect tissues. Based on this fact,

EPNs may reasonably affect the nutritional indices and growth rates of infected insects.

The obtained data of our semi-field trials during two growing seasons of cotton crop against the 4th instars of *S. littoralis* showed high mortality percentages in *S. carpocapsae* at field rate (≡ LC_{90}) more than *H. indica* from the 3rd to the 9th DAT, in season 2021. However, both of them showed equipollent toxic effects along the 12th DATs, in season 2022. These findings came in accordance with the data of susceptibility test carried out by Taha (2021) that exhibited potent lethal effects on the 5th instar larvae of *S. littoralis* treated by the range of 200 up to 1600 IJs of each of *S. carocapsae* and *H. indica* after 48 hrs. Nevertheless, Khan *et al.* (2021) found that the mortality responses in *S. litura* to 500 IJs of each of *S. carocapsae* and *H. indica* at 72 hrs were limited at 38.00 and 44.00 %, respectively. Meantime, the selected binary mixture of the tested EPNs at field rate (≡ LC_{50} realized highest additive effect) transcends all the individual treatments of EPN at rates ≡ LC_{90} along the 12th DATs. Eventually, the results of overall mean of mortality percentages of the binary mixture of EPNs excelled *S. carpocapsae* while *H. indica* came last. Likewise, the LT_{50} of the binary mixture of EPNs overpassed the tested EPNs alone. Here, we have to point out that many species belong to the main genera of symbiotic bacteria, *Photorhabdus* and *Xenorhabdus* specified for *S. carpocapsae* as well as *Photorhabdus* for *H. indica* secrete proteases, protoxins and some insecticidal complexes that play important role in actin polymerization and destabilization in haemocytes of insects (Lalitha *et al*., 2018; Neira-Monsalve *et al*., 2020). These secondary metabolites also generate reactive oxygen species that initiate distractive reactions on cells and tissues ending with fatal septicemia within 2 or 3 days (Lalitha *et al*., 2018; Huot *et al*., 2019; Neira-Monsalve *et al*., 2020). Thus, the observed superiority of the binary mixture of the tested EPNs in our study may be attributed to the possibility of increases in diversity and multiplicity of the symbiotic species as well as to their secondary metabolites that may act synergistically on the efficacy of the binary mixture against the treated larvae of *S. littoralis*. This assumption was deduced from many publications discussed the additive or synergistic effects produced by the interaction of symbiotic bacteria of EPNs or even the secondary metabolites with other bio-agents against insect pests or diseases (Jung and Kim, 2006; Abd-Elgawad, 2021; Kusakabe *et al*., 2022). This speculation could be a novel starting point for prospective studies.

Phenoloxidase (PO) activity:

In the current investigation, we assessed the correlation between the pathogenicity of two different strains of EPNs and PO activity levels in *S. littoralis* fourth instar. In comparison to the control, the *H. indica* showed an increase in enzymatic activity of 62.7% after 48 hrs of infection, followed by 52.9% for *S. carpocapsae* and 21.6% of the binary mixture of the tested EPNs. Prophenoloxidase (PPO) is an inactive proenzyme of (PO), which are activated by a protease cascade, it plays important roles in the development of nodules, wound healing, and cuticular tanning. In order to get rid of parasites and pathogens that are entering the body, PO catalyses the biosynthesis of quinones and other reactive intermediates, which plays a part in the processes of encapsulation and melanisation (Lokstan and Li, 1988). Numerous researchers have examined the PO activity, purification, and characterization in various insects, *Apis mellifera* (Zufelato *et al*., 2004), *Manduca sexta* (Hall *et al*., 1995), *Eurygaster integriceps* (Zibaee *et al*., 2011). *Drosophila melanogaster* (Sezaki *et al*., 2001), *Bombyx mori* (Yasuhara *et al*., 1995, Liu *et al*., 2013) *Helicoverpa armigera* (Goudru *et al*., 2013). PO is one of the most crucial defensive mechanisms used by invertebrate to defend themselves from invading pathogen threats such as nematodes (Brivio *et al*., 2002), multiple nucleopolyhedrovirus (Kasianov *et al*., 2017), parasitoids and fungal pathogens (Hartzer *et al*., 2005; Kong *et al*., 2013; Zhao *et al*., 2007). According to SDS-PAGE results, PO is made up of a polypeptide chain with a relative molecular weight of less than 80 kDa (Ashida, 1971; Hall *et al*, 1995; Rajagopal *et al*., 2005; Goudru *et al*., 2013). Consistent with our findings, in nematode plasma larvae, an extra band with a molecular weight of ~79 was seen after 48 hrs of PIPs, we hypothesized that the PO was activated after infection. It has been shown that endoparasitoid and entomopathogenic nematode infections in lepidopteran hosts accelerate the induction of PO activity (Yokoo *et al*., 1992; Vinson, 197ו). However, current result has been demonstrated that EPNs binary combinations suppress PO activity in the tested hemolymph also, in a molecular weight intensity of ~79. In keeping with previous studies demonstrated that one of a parasite's main strategies for defeating its host is a decrease in immune responses and PO level activity (Dunphy and Webster, 1984; Ribeiro *et al*., 1999; Brivio *et al*., 2002). This enzyme's transcript level rises in response to parasite invasion, and insects whose production of this enzyme is disrupted are less able to enclose and melanize pathogens to impede their attack (Shiao *et al*., 2001).

Quantitative and qualitative analysis of *S. littoralis* hemolymph protein:

In this study, total hemolymph protein (THPs) contents of larval infected with the binary mixing of the EPNs, were significantly reduced by 2.8 times compared to the uninfected (control group) larvae.

While the haemolymph protein concentrations of *H. indica* and *S. carpocapsae* larvae were 1.3 and 1.8 times lower than control, respectively. This decline may be caused by the development of the entomopathogenic, when the IJs received nutrients from their host body two days of PIPs. This conclusion was consistent with previous effects of the parasite's reaction to the host; the parasite *Cotesia glomerata* benefited from the host's provision of specific nutrients during different phases of its development (Vinson, 1976). The pathogenicity of *Steinernema* species was observed with the entry of IJs into the bodies of insect hosts, and this was corroborated by the decrease in hemolymph protein samples after 24 hours (Istkhar and Chaubey, 201^). A decrease in total protein was also seen after *S. littoralis* larvae were treated with insect growth regulators (IGRs) such Diflubenzuron and chromafenozide (Saleh and Abdel-Gawad, 2018). The lower protein concentration could be the result of DNA damage turning off some crucial genes that produce this protein, inhibiting DNA synthesis, or a decline in the activity of various enzymes (Hamouda, 2002). Same results of THPs of *S. littoralis* injected with Staphylococcal enterotoxin A (SEA) were drastically reduced considerably after 48 hrs; this is likely due to the extensive consumption of plasma proteins during bacterial multiplication and growth (Radwan *et al*., 2022). Another study found that *Mertarhizium anisopliae* infection reduced the total hemolymph protein content of *Schistocerca gregaria* (Gillespie *et al*., 2000).

A considerable subunits difference between control and infected larvae were seen in the hemolymph protein profile of *S. littoralis* larvae. For instance, there was a new synthesis of proteins of ~79 KDa molecular weight, and some bands were weaker or stronger compared to those seen in normal larvae. This result was consistent with earlier research where injections of tiny amounts of the Teratocyte Secreted Proteins (TSP) from *Microplitis Croceipes* into the haemolymph of *Heliothis virescens* caused the synthesis of specific 70-80 kDa proteins (Järlfors *et al*., 1997). The major protein components of the infective larvae of *S. carocapsae* and *H. indica* were seen to have significantly decreased in intensity, in line with the drop in the total hemolymph protein level compared to control. These findings are consistent with those of Schmidt and Platzer (1980), who found protein breakdown in *Culex pipiens* after infection with *Romanomermis culicivorax*, they proposed the degradation of *C. pipiens* hemolymph proteins occurs as a result of some proteases produced by nematodes. According to Wee *et al.* (2000), after the nematode-bacterium complex enters, bacterial cells produce proteases which then break down the protein of insects and provide food resources for the nematode growth. Analysis of the haemolymph proteins in *S. littoralis* revealed that the host not only included a 212 kDa protein that was lacking in non-parasitized larvae, but also a significant protein band of 70 kDa that had emerged one instar earlier in parasitized larvae (Lanzrein *et al*., 1998). At 9, 12, and 24 hrs after infection, a new band with a molecular weight of around 28 and 46 kDa formed in the hemolymph plasma of *Helicoverpa armigera* larvae, this band was present as a result of the entry of the entomopathogenic nematode *Steinernema abbasi* into the host body, they hypothesised that the release of major hemolymph proteins and some unidentified proteins by hemocytes may aid in initial host defence and prevent parasite invasion (Istkhar and Chaubey, 2018). Other researchers backed up the study in which it was stated that *Steinernema carpocapsae* and bacteria produced proteases, which led to a subsequent decrease in proteins (Toubarro *et al*., 2013). Surprisingly, the binary mixture of the studied EPNs had less quantitative hemolymph protein than the individual infection, according to this data. However, the number and proportion of protein fractions as determined by electrophoretic analysis increased greatly, especially for proteins with molecular weights ≤ 30 KDa. This would suggest a connection between the synthesis of AMPs and bacterial-nematode invasion. AMPs destruct the pathogen by different mechanisms, including disrupting cell membranes, inhibiting bacterial metabolism, as well as destroy the components of the cytoplasm (Shen *et al*., 2018). In line with earlier findings, remark that the synthesis of novel AMPs with a molecular mass under 30 kDa, which related in immune responses and the deadly consequences of certain bacterial strains released by nematodes after penetration (Gillespie *et al*., 1997; Bulet and Stocklin, 2005).

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الملخص العربي

الاستجابة المناعية وتقييم الكفاءة الحيوية لديدان النيماتودا الممرضة للحشرات كمبيد حيوي لدودة ورق القطن

ريما شاهين، وائل محمود خميس، أحمد الصبروت

تهدف الدراسة الحالية إلى التحقيق في السمية المتبقية والمؤشرات الغذائية للنيماتودا الممرضة للحشرات (EPNs) لدودة ورق القطن *Spodoptera littoralis*، وكذلك الاستجابة المناعية للعدوى. مقارنة بالتأثيرات الفردية للنيماتودا الممرضة للحشرات *Steinernema carpocapsae* و *Heterorhabditis indica*، وجد أن التوليفات الثنائية لكلا منهما تكون أكثر إمراضيه لدودة ورق القطن *S. littoralis* وذلك وفقًا لنسب الموت والاستجابات الفسيولوجية والمناعية. بينما كان نسب النشاط المضاد للتغذية في يرقات دودة ورق القطن المصابة *S. carpocapsae* و *H. indica* ، تأثير أقوى من الخليط الثنائي عند LC_{35}، مقارنة بنشاط التغذية الكامل في اليرقات السليمة. أظهرت الخصائص الكيميائية الحيوية للدم في يرقات دودة ورق القطن المصابة تباينًا معنويًا، ليس فقط في تركيز البروتين الكلي، ولكن أيضًا في تخليق بروتينات جديدة. حيث لوحظ وجود بروتين جديد، بوزن جزيئي يبلغ ٧٩ كيلو دالتون، افترضنا أنه قد يكون انريم الفينولوكسيديز (PO)، والذي يتزامن بشكل وثيق مع الحث الكبير لنشاط PO بعد الإصابة. ومن المثير للاهتمام، أن دم اليرقات المصابة بتوليفات ثنائية من EPNs أظهر زيادة ملحوظة في كثافة البروتينات الرئيسية وزيادة في عدد الببتيدات المضادة للميكروبات ذات الأوزان الجزيئية التي تقل عن ٣٠ كيلو دالتون. ومع ذلك، فإن الانخفاض الكبير في تركيز البروتين الكلي ومستوى PO ، والذي يؤدي في النهاية إلى موت اليرقات، هو دليل على أن المزيج الثنائي من الديدان الخيطية تثبط وبشدة الجهاز المناعي ليرقات دودة ورق القطن *S. littoralis*

الكلمات المفتاحية: النيماتودا الممرضة للحشرات، الاستجابة المناعية الخلطية، تقييم الكفاءة الحيوية، *Spodoptera littoralis*.